Polymer Conjugates of Cytokines, Chemokines, Growth Factors, Polypeptide Hormones and Antagonists Thereof with Preserved Receptor-Binding Activity

CROSS REFERENCE TO RELATED APPLICATIONS

[0001] The present application claims the benefit of the filing dates of U.S. Provisional Appl. No. 60/479,914, filed June 20, 2003, and U.S. Provisional Application No. 60/436,020, filed December 26, 2002. The disclosures of the above-referenced applications are incorporated herein by reference in their entireties.

BACKGROUND OF THE INVENTION

Field of the Invention

[0002] The present invention is in the fields of protein biochemistry and the pharmaceutical and medical sciences. In particular, the invention provides methods for the production of conjugates between water-soluble polymers (*e.g.*, poly(ethylene glycol) and derivatives thereof) and certain bioactive components, which conjugates have increased receptor-binding activity compared to standard polymer-bioactive component conjugates. More specifically, the invention provides methods for the production of polymer conjugates of certain receptor-binding proteins with unusually high receptor-binding activity. The invention also provides conjugates produced by such methods, compositions comprising such conjugates, kits comprising such conjugates and compositions and methods of use of the conjugates and compositions in preventing, diagnosing and treating a variety of medical and veterinary conditions.

Related Art

[0003] The following description of related art includes interpretations of the present inventors that are not, themselves, in the prior art. Cytokines are secreted regulatory proteins that control the survival, growth, differentiation, and/or effector function of cells in endocrine, paracrine or autocrine fashion

(reviewed in Nicola, N.A. (1994) *in: Guidebook to Cytokines and Their Receptors*, Nicola, N.A., ed., pp. 1-7, Oxford University Press, New York). Chemokines are a family of structurally related glycoproteins with potent leukocyte activation and/or chemotactic activities (reviewed in Oppenheim, J.J., *et al.*, (1997) *Clin Cancer Res 3*:2682-2686). Like their close relatives, the polypeptide hormones and growth factors, cytokines and chemokines initiate their regulatory functions by binding to specific receptor proteins on the surface of their target cells (reviewed in Kossiakoff, A.A., *et al.*, (1998) *Adv Protein Chem 52*:67-108; Onuffer, J.J., *et al.*, (2002) *Trends Pharmacol Sci 23*:459-467). Because of their potency, specificity, small size and relative ease of production in recombinant organisms, cytokines, chemokines, growth factors and polypeptide hormones have many potential applications as therapeutic agents. Two key factors have hindered the development of cytokines, in particular, and recombinant proteins, in general, as therapeutic agents – their generally short half-lives in the circulation and their potential antigenicity and immunogenicity. As used herein and generally in the art, the term "antigenicity" refers to the ability of a molecule to bind to preexisting antibodies, while the term "immunogenicity" refers to the ability of the molecule to evoke an immune response *in vivo*, whether that response involves the formation of antibodies (a "humoral response") or the stimulation of cellular immune responses.

[0004] For the administration of recombinant therapeutic proteins, intravenous (*i.v.*) administration is often desirable in order to achieve the highest circulating activities and to minimize problems of bioavailability and degradation. However, the half-lives of small proteins following *i.v.* administration are usually extremely short (see examples in Mordenti, J., *et al.*, (1991) *Pharm Res 8*:1351-1359; Kuwabara, T., *et al.*, (1995) *Pharm Res 12*:1466-1469). Proteins with hydrodynamic radii exceeding that of serum albumin, which has a Stokes radius of about 36 Å and a molecular weight of about 66,000 Daltons (66 kDa), are generally retained in the bloodstream by healthy kidneys. However, smaller proteins, including cytokines such as

granulocyte colony-stimulating factor ("G-CSF"), interleukin-2 ("IL-2"), interferon-*alpha* ("IFN-*alpha*") and interferon-*gamma* ("IFN-*gamma*"), are cleared rapidly from the bloodstream by glomerular filtration (Brenner, B.M., *et al.*, (1978) *Am J Physiol 234*:F455-F460; Venkatachalam, M.A. *et al.*, (1978) *Circ Res 43*:337-347; Wilson, G., (1979) *J Gen Physiol 74*:495-509; Knauf, M.J., *et al.*, (1988) *J Biol Chem 263:*15064-15070; Kita, Y., *et al.*, (1990) *Drug Des Deliv 6*:157-167; Rostaing, L., *et al.*, (1998), *J Am Soc Nephrol 9*:2344-2348). As a result, the maintenance of therapeutically useful concentrations of small recombinant proteins in the circulation is problematic following injection. Therefore, higher concentrations of such proteins and more frequent injections typically must be administered. The resulting dose regimens increase the cost of therapy, decrease the likelihood of patient compliance and increase the risk of adverse events, *e.g.*, immune reactions. Both cellular and humoral immune responses can reduce the circulating concentrations of injected recombinant proteins to an extent that may preclude the administration of an effective dose or may lead to treatment-limiting events including accelerated clearance, neutralization of efficacy and anaphylaxis (Ragnhammar, P., *et al.*, (1994) *Blood 84*:4078-4087; Wadhwa, M., *et al.*, (1999) *Clin Cancer Res 5*:1353-1361; Hjelm Skog, A.-L., *et al.*, (2001) *Clin Cancer Res 7*:1163-1170; Li, J., *et al.*, (2001) *Blood 98*:3241-3248; Basser, R.L., *et al.*, (2002) *Blood 99*:2599-2602; Schellekens, H., (2002) *Clin Ther 24*:1720-1740).

[0005] Modification of recombinant proteins by the covalent attachment of poly(ethylene glycol) ("PEG") has been investigated extensively as a means of addressing the shortcomings discussed above (reviewed in Sherman, M.R., *et al.*, (1997) *in*: *Poly(ethylene glycol): Chemistry and Biological Applications*, Harris, J.M., *et al.*, eds., pp. 155-169, American Chemical Society, Washington, D.C.; Roberts, M.J., *et al.*, (2002) *Adv Drug Deliv Rev 54*:459-476). The attachment of PEG to proteins has been shown to stabilize the proteins, improve their bioavailability and/or reduce their immunogenicity *in vivo*. (The covalent attachment of PEG to a protein or other substrate is

referred to herein, and is known in the art, as "PEGylation.") In addition, PEGylation can increase the hydrodynamic radius of proteins significantly. When a small protein, such as a cytokine, chemokine, growth factor or polypeptide hormone, is coupled to a single long strand of PEG (*e.g.* having a molecular weight of at least about 18 kDa), the resultant conjugate has a hydrodynamic radius exceeding that of serum albumin and its clearance from the circulation *via* the renal glomeruli is retarded dramatically. The combined effects of PEGylation – reduced proteolysis, reduced immune recognition and reduced rates of renal clearance – confer substantial advantages on PEGylated proteins as therapeutic agents.

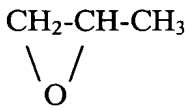
[0006] Since the 1970s, attempts have been made to use the covalent attachment of polymers to improve the safety and efficacy of various proteins for pharmaceutical use (see, *e.g.*, Davis, F.F., *et al.*, U.S. Patent No. 4,179,337). Some examples include the coupling of PEG or poly(ethylene oxide) ("PEO") to adenosine deaminase (EC 3.5.4.4) for use in the treatment of severe combined immunodeficiency disease (Davis, S., *et al.*, (1981) *Clin Exp Immunol 46*:649-652; Hershfield, M.S., *et al.*, (1987) *N Engl J Med 316*:589-596), to superoxide dismutase (EC 1.15.1.1) for the treatment of inflammatory conditions (Saifer, M., *et al.*, U.S. Patent Nos. 5,006,333 and 5,080,891) and to urate oxidase (EC 1.7.3.3) for the elimination of excess uric acid from the blood and urine (Kelly, S.J., *et al.*, (2001) *J Am Soc Nephrol 12*:1001-1009; Williams, L.D., *et al.*, PCT Publication No. WO 00/07629 A2 and A3 and U.S. Patent No. 6,576,235; Sherman, M.R., *et al.*, PCT Publication No. WO 01/59078 A2).

[0007] PEOs and PEGs are polymers composed of covalently linked ethylene oxide units. These polymers have the following general structure:

$$R_1\text{-}(OCH_2CH_2)_n\text{-}R_2$$

where R_2 may be a hydroxyl group (or a reactive derivative thereof) and R_1 may be hydrogen, as in dihydroxyPEG ("PEG diol"), a methyl group, as in monomethoxyPEG ("mPEG"), or another lower alkyl group, *e.g.*, as in *iso*-propoxyPEG or *t*-butoxyPEG. The parameter *n* in the general structure of

PEG indicates the number of ethylene oxide units in the polymer and is referred to herein and in the art as the "degree of polymerization." Polymers of the same general structure, in which R_1 is a C_{1-7} alkyl group, have also been referred to as oxirane derivatives (Yasukohchi, T., *et al.*, U.S. Patent No. 6,455,639). PEGs and PEOs can be linear, branched (Fuke, I., *et al.*, (1994) *J Control Release 30*:27-34) or star-shaped (Merrill, E.W., (1993) *J Biomater Sci Polym Ed 5*:1-11). PEGs and PEOs are amphipathic, *i.e.* they are soluble in water and in certain organic solvents and they can adhere to lipid-containing materials, including enveloped viruses and the membranes of animal and bacterial cells. Certain random or block or alternating copolymers of ethylene oxide (OCH_2CH_2) and propylene oxide, which has the following structure:



have properties that are sufficiently similar to those of PEG that these copolymers are thought to be suitable replacements for PEG in certain applications (see, *e.g.*, Hiratani, H., U.S. Patent No. 4,609,546 and Saifer, M., *et al.*, U.S. Patent No. 5,283,317). The term "polyalkylene oxides" and the abbreviation "PAOs" are used herein to refer to such copolymers, as well as to PEGs or PEOs and to poly(oxyethylene-oxymethylene) copolymers (Pitt, C.G., *et al.*, U.S. Patent No. 5,476,653). As used herein, the term "polyalkylene glycols" and the abbreviation "PAGs" are used to refer generically to polymers suitable for use in the conjugates of the invention, particularly PEGs, more particularly PEGs containing a single reactive group ("monofunctionally activated PEGs").

[0008] The covalent attachment of PEG or other polyalkylene oxides to a protein requires the conversion of at least one end group of the polymer into a reactive functional group. This process is frequently referred to as "activation" and the product is called "activated PEG" or activated polyalkylene oxide. MonomethoxyPEGs, in which an oxygen at one end is capped with an unreactive, chemically stable methyl group (to produce a

"methoxyl group") and on the other end with a functional group that is reactive towards amino groups on a protein molecule, are used most commonly for such approaches. So-called "branched" mPEGs, which contain two or more methoxyl groups distal to a single activated functional group, are used less commonly. An example of branched PEG is di-mPEG-lysine, in which PEG is coupled to both amino groups, and the carboxyl group of lysine is most often activated by esterification with *N*-hydroxysuccinimide (Martinez, A., *et al.*, U.S. Patent No 5,643,575; Greenwald, R.B., *et al.*, U.S. Patent No. 5,919,455; Harris, J.M., *et al.*, U.S. Patent No. 5,932,462).

[0009] Commonly, the activated polymers are reacted with a bioactive compound having nucleophilic functional groups that serve as attachment sites. One nucleophilic functional group that is used commonly as an attachment site is the *epsilon* amino group of lysine residues. Solvent-accessible *alpha*-amino groups, carboxylic acid groups, guanidino groups, imidazole groups, suitably activated carbonyl groups, oxidized carbohydrate moieties and thiol groups have also been used as attachment sites.

[0010] The hydroxyl group of PEG has been activated with cyanuric chloride prior to its attachment to proteins (Abuchowski, A., *et al.*, (1977) *J Biol Chem 252*:3582-3586; Abuchowski, A., *et al.*, (1981) *Cancer Treat Rep 65*:1077-1081). The use of this method has disadvantages, however, such as the toxicity of cyanuric chloride and its non-specific reactivity for proteins having functional groups other than amines, such as solvent-accessible cysteine or tyrosine residues that may be essential for function. In order to overcome these and other disadvantages, alternative activated PEGs have been introduced, such as succinimidyl succinate derivatives of PEG ("SS-PEG") (Abuchowski, A., *et al.*, (1984) *Cancer Biochem Biophys 7*:175-186), succinimidyl carbonate derivatives of PAG ("SC-PAG") (Saifer, M., *et al.*, U.S. Patent No. 5,006,333) and aldehyde derivatives of PEG (Royer, G.P., U.S. Patent No. 4,002,531).

[0011] Commonly, several (*e.g.*, 5 to 10) strands of one or more PAGs, *e.g.*, one or more PEGs with a molecular weight of about 5 kDa to about 10 kDa,

are coupled to the target protein *via* primary amino groups (the *epsilon* amino groups of lysine residues and, possibly, the *alpha* amino group of the amino-terminal ("N-terminal") amino acid). More recently, conjugates have been synthesized containing a single strand of mPEG of higher molecular weight, *e.g.,* 12 kDa, 20 kDa or 30 kDa. Direct correlations have been demonstrated between the plasma half-lives of the conjugates and an increasing molecular weight and/or increasing number of strands of PEG coupled (Knauf, M.J., *et al., supra*; Katre, N.V. (1990) *J Immunol 144*:209-213; Clark, R., *et al.,* (1996) *J Biol Chem 271*:21969-21977; Leong, S.R., *et al.,* (2001) *Cytokine 16*:106-119). On the other hand, as the number of strands of PEG coupled to each molecule of protein is increased, so is the probability that an amino group in an essential region of the protein will be modified and hence the biological function of the protein will be impaired, particularly if it is a receptor-binding protein. For larger proteins that contain many amino groups, and for enzymes with substrates of low molecular weight, the tradeoff between increased duration of action and decreased specific activity may be acceptable, since it produces a net increase in the biological activity of the PEG-containing conjugates *in vivo*. For smaller proteins that function *via* interactions with cell-surface receptors, such as cytokines, chemokines, growth factors and polypeptide hormones, however, a relatively high degree of substitution has been reported to decrease the functional activity to the point of negating the advantage of an extended half-life in the bloodstream (Clark, R., *et al., supra*).

[0012] Thus, polymer conjugation is a well-established technology for prolonging the bioactivity and decreasing the immunoreactivity of therapeutic proteins such as enzymes (*see, e.g.*, U.S. Provisional Appl. No. 60/436,020, filed December 26, 2002, and U.S. Provisional Appl. Nos. 60/479,913 and 60/479,914, both filed on June 20, 2003, the disclosures of which are incorporated herein by reference in their entireties). However, the conjugation of polymers to receptor-binding proteins that function by binding specifically to cell-surface receptors usually: 1) interferes with such binding; 2) markedly diminishes the signal transduction potencies of cytokine, chemokine, growth

factor and polypeptide hormone agonists; and 3) markedly diminishes the competitive potencies of cytokine, chemokine, growth factor and polypeptide hormone antagonists. Published examples of such conjugates with diminished receptor-binding activity include polymer conjugates of human growth hormone ("hGH") (Clark, R., *et al., supra*), hGH antagonists (Ross, R.J.M., *et al.*, (2001) *J Clin Endocrinol Metab 86*:1716-1723; IFN-*alpha* (Bailon, P., *et al.*, (2001) *Bioconjug Chem 12*:195-202; Wylie, D.C., *et al.*, (2001) *Pharm Res 18*:1354-1360; Wang, Y.-S., *et al.*, (2002) *Adv Drug Deliv Rev 54*:547-570) and G-CSF (Kinstler, O., *et al.*, PCT Publication No. WO 96/11953; Bowen, S., *et al.*, (1999) *Exp Hematol 27*:425-432), among others. In an extreme case, the coupling of polymers to interleukin-15 ("IL-15") converted this IL-2-like growth factor into an inhibitor of cellular proliferation (Pettit, D.K., *et al.*, (1997) *J Biol Chem 272*:2312-2318). While not intending to be bound by theory, the mechanism for such undesirable effects of PEGylation may involve steric hindrance of receptor interactions by the bulky PEG groups, charge neutralization, or both.

[0013] Thus, there exists a need for methods for producing PAG-containing (*e.g.*, PEG- and/or PEO-containing) conjugates, particularly conjugates between such water-soluble polymers and receptor-binding proteins, with preservation of substantial bioactivity (*e.g.*, at least about 40%), nearly complete bioactivity (*e.g.*, at least about 80%) or essentially complete bioactivity (*e.g.*, at least about 90%). Such conjugates will have the benefits provided by the polymer component of increased solubility, stability, half-life and bioavailability *in vivo* and will exhibit substantially increased potency or utility, compared to conventional polymer conjugates, in an animal into which the conjugates have been introduced for prophylactic, therapeutic or diagnostic purposes.

BRIEF SUMMARY OF THE INVENTION

[0014] The present invention addresses the needs identified above, and provides methods for the preparation of conjugates of water-soluble polymers,

e.g., poly(ethylene glycol), and derivatives thereof, with bioactive components, especially receptor-binding proteins, particularly therapeutic or diagnostic bioactive components such as cyokines, chemokines, polypeptide hormones and polypeptide growth factors. The invention also provides conjugates produced by such methods. Compared to the corresponding unconjugated bioactive components, the conjugates of the invention have increased stability (*i.e.*, longer shelf life and longer half-lives *in vivo*). In addition, compared to conjugates of the same bioactive component prepared with polymer chains that are attached randomly to solvent-accessible sites along the polypeptide chains, the conjugates of the invention have increased receptor-binding activity, which can be measured or employed *in vitro,* and increased potency *in vivo*. The invention also provides such improved conjugates for use in industrial cell culture. Furthermore, the invention provides compositions comprising such conjugates, kits containing such conjugates and compositions and methods of use of the conjugates and compositions in a variety of prophylactic, diagnostic and therapeutic regimens.

[0015] In one embodiment, the invention provides methods for preserving the receptor-binding potency of a cytokine, a chemokine, a growth factor or a polypeptide hormone, comprising selectively coupling one or more synthetic water-soluble polymers to the amino-terminal amino acid of the cytokine, chemokine, growth factor or polypeptide hormone, or an antagonist thereof, wherein the amino-terminal amino acid is located remotely from one or more receptor-binding domains of the cytokine, chemokine, growth factor or polypeptide hormone, or antagonist thereof. In a related embodiment, the invention provides methods for preserving the receptor-binding potency of a cytokine, a chemokine, a growth factor and a polypeptide hormone, or an antagonist thereof, comprising selectively coupling one or more synthetic water-soluble polymers at or near one or more glycosylation sites of the cytokine, chemokine, growth factor or polypeptide hormone, or antagonist thereof, wherein the one or more glycosylation sites is/are located remotely

from one or more receptor-binding domains of the cytokine, chemokine, growth factor or polypeptide hormone.

[0016] Suitable polymers for use in these methods of the invention include, but are not limited to, one or more polyalkylene glycols (including, but not limited to, one or more poly(ethylene glycols), one or more monomethoxy-poly(ethylene glycols) and one or more monohydroxypoly(ethylene glycols)), one or more polyalkylene oxides, one or more polyoxiranes, one or more polyolefinic alcohols, *e.g.*, polyvinyl alcohol, one or more polycarboxylates, one or more poly(vinylpyrrolidones), one or more poly(oxyethylene-oxymethylenes), one or more poly(amino acids), one or more polyacryloyl-morpholines, one or more copolymers of one or more amides and one or more alkylene oxides, one or more dextrans and one or more hyaluronic acids. Polymers suitable for use in the methods of the invention typically have molecular weights of between about 1 kDa and about 100 kDa, inclusive, or more particularly molecular weights of between about 1 kDa and about 5 kDa, inclusive; between about 10 kDa and about 20 kDa, inclusive; between about 18 kDa and about 60 kDa, inclusive; between about 12 kDa and about 30 kDa, inclusive; or of about 10kDa, about 20 kDa or about 30 kDa.

[0017] A variety of cytokines, chemokines, growth factors and polypeptide hormones (and analogs that mimic (*i.e.*, agonize) or antagonize the biological effects of the corresponding cytokine, chemokine, growth factor or polypeptide hormone that are mediated by their specific cell-surface receptors) are suitable for use in preparing the present conjugates. These include cytokines, chemokines, growth factors or polypeptide hormones having a four helix bundle structure (including but not limited to granulocyte colony-stimulating factor (G-CSF), macrophage colony-stimulating factor (M-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), leukemia inhibitory factor (LIF), erythropoietin (Epo), thrombopoietin (Tpo), stem cell factor (SCF), Flt3 ligand, oncostatin M (OSM), interleukin-2 (IL-2), IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-11, IL-12 (p35 subunit), IL-13, IL-15, IL-17, interferon *alpha* (IFN-α), interferon *beta* (IFN-β) (including IFN-β-1b),

consensus interferon, prolactin and growth hormone, and muteins, variants, analogs and derivatives thereof); cytokines, chemokines, growth factors or polypeptide hormones having a β-sheet or β-barrel structure (including but not limited to tumor necrosis factor-*alpha* (TNF-α), IL-1α, IL-1β, IL-12 (p40 subunit), IL-16, epidermal growth factor (EGF), insulin-like growth factor 1 (IGF-1), basic fibroblast growth factor (bFGF), acidic FGF, FGF-4 and keratinocyte growth factor (KGF; FGF-7), and muteins, variants, analogs and derivatives thereof); and cytokines, chemokines, growth factors or polypeptide hormones having a mixed α/β structure (including but not limited to neutrophil activating peptide-2 (NAP-2), stromal cell-derived factor-1α (SDF-1α), IL-8, monocyte chemoattractant protein-1 (MCP-1), MCP-2, MCP-3, eotaxin-1, eotaxin-2, eotaxin-3, RANTES, myeloid progenitor inhibitory factor-1 (MPIF-1), neurotactin, macrophage migration inhibitory factor (MIF) and GRO/melanoma growth stimulatory activity (GRO-α/MGSA), and muteins, variants, analogs and derivatives thereof). Polypeptide hormones suitable for use in the present invention include, but are not limited to, insulin and insulin analogs that mimic or antagonize the biological effects of insulin that are mediated by insulin receptors; prolactin and prolactin analogs that mimic or antagonize the biological effects of prolactin that are mediated by prolactin receptors; and growth hormone (particularly human growth hormone) and analogs thereof that mimic or antagonize the biological effects of growth hormone that are mediated by growth hormone receptors.

[0018] Particularly preferred cytokines, chemokines, growth factors and polypeptide hormones suitable for use in accordance with the present invention include IL-2; IL-10; IFN-α; IFN-β (including IFN-β-1b); TNF-*alpha*; IGF-1; EGF; bFGF; hGH; prolactin; and insulin. Also particularly suitable for use are competitive antagonists of the foregoing cytokines, chemokines, growth factors and polypeptide hormones, *e.g.*, antagonists of TNF-*alpha*, hGH or prolactin, as well as muteins, variants and derivatives of these cytokines, chemokines, growth factors and polypeptide hormones.

[0019] In certain embodiments, the one or more polymers is/are covalently coupled (particularly *via* a secondary amine linkage) to the *alpha* amino group of the amino-terminal amino acid on the cytokine, chemokine, growth factor or polypeptide hormone. In other embodiments, the one or more polymers is/are covalently coupled to a chemically reactive side chain group (*e.g.*, a hydroxyl group, a sulfhydryl group, a guanidino group, an imidazole group, an amino group, a carboxyl group or an aldehyde derivative) of the amino-terminal amino acid on the cytokine, chemokine, growth factor or polypeptide hormone. In additional embodiments, the coupling of the polymer to the cytokine, chemokine, growth factor or polypeptide hormone at the amino-terminal amino acid or at or near one or more glycosylation sites mimics the beneficial effects of glycosylation of the cytokine, chemokine, growth factor or polypeptide hormone. In related embodiments, the coupling of the polymer to the cytokine, chemokine, growth factor or polypeptide hormone at or near one or more glycosylation sites on the cytokine, chemokine, growth factor or polypeptide hormone mimics the beneficial effects of hyperglycosylation of the cytokine, chemokine, growth factor or polypeptide hormone, wherein "hyperglycosylation" indicates the covalent attachment of simple or complex carbohydrate moieties in addition to those present in the native structure.

[0020] The invention also provides conjugates produced by the methods of the invention. Conjugates of the invention comprise a selected cytokine, a selected chemokine, a selected growth factor, a selected polypeptide hormone or a selected antagonist thereof (such as those described above) coupled to one or more synthetic water-soluble polymers (such as those described above), wherein the one or more polymers is/are coupled to the amino-terminal amino acid of the cytokine, chemokine, growth factor or polypeptide hormone, and wherein the amino-terminal amino acid is located remotely from one or more receptor-binding domains of the selected cytokine, chemokine, growth factor or polypeptide hormone. Additionally, conjugates of the invention comprise a selected cytokine, a selected chemokine, a selected growth factor or a selected polypeptide hormone, or a selected antagonist thereof (such as those described

above), coupled to one or more synthetic water-soluble polymers (such as those described above), wherein the one or more polymers is/are coupled to one or more glycosylation sites of the selected cytokine, chemokine, growth factor or polypeptide hormone, or antagonist thereof, and wherein the one or more glycosylation sites is/are located remotely from one or more receptor-binding domains of the cytokine, chemokine, growth factor or polypeptide hormone, or antagonist thereof. For polymer conjugates of agonists of the invention, it is preferable that the site(s) of polymer attachment be remote from all of the receptor-binding domains. For polymer conjugates of certain antagonists of the invention, it may be preferable that the site(s) of polymer attachment be remote from certain receptor-binding domains that are essential for binding to occur, but not necessarily remote from all of the receptor-binding domains that are essential for signal transduction by agonists. The invention also provides compositions, particularly pharmaceutical compositions, comprising one or more of the conjugates of the invention and one or more additional components, such as one or more pharmaceutically acceptable diluents, excipients or carriers. The invention also provides kits comprising one or more of the conjugates, compositions and/or pharmaceutical compositions of the invention.

[0021] The invention also provides methods of preventing, diagnosing, or treating a physical disorder in an animal (*e.g.*, a mammal such as a human) suffering from or predisposed to the physical disorder. Such methods may comprise, for example, administering to the animal an effective amount of one or more of the conjugates, compositions or pharmaceutical compositions of the present invention. Physical disorders suitably treated or prevented according to such methods of the invention include, but are not limited to, cancers (*e.g.*, a breast cancer, a uterine cancer, an ovarian cancer, a prostate cancer, a testicular cancer, a lung cancer, a leukemia, a lymphoma, a colon cancer, a gastrointestinal cancer, a pancreatic cancer, a bladder cancer, a kidney cancer, a bone cancer, a neurological cancer, a head and neck cancer, a skin cancer, a sarcoma, an adenoma, a carcinoma and a myeloma); infectious

diseases (*e.g*, bacterial diseases, fungal diseases, parasitic diseases and viral diseases (such as a viral hepatitis, a disease caused by a cardiotropic virus; HIV/AIDS; and the like)); and genetic disorders (*e.g.*, anemia, neutropenia, thrombocytopenia, hemophilia, dwarfism and severe combined immunodeficiency disease ("SCID"); autoimmune disorders (*e.g.*, psoriasis, systemic lupus erythematosus and rheumatoid arthritis) and neurodegenerative disorders (*e.g.*, various forms and stages of multiple sclerosis, Creutzfeldt-Jakob Disease, Alzheimer's Disease, and the like).

[0022] Other preferred embodiments of the present invention will be apparent to one of ordinary skill in light of the following drawings and description of the invention, and of the claims.

BRIEF DESCRIPTION OF THE DRAWINGS

[0023] Figures 1 through 8 display molecular models of various cytokines and growth factors created with RasMol software (Sayle, R.A., *et al.,* (1995) *Trends Biochem Sci 20*:374-376) based on crystallographic data. Each of the models is represented in "ribbon" or "cartoon" format, except for certain residues of particular interest, which are shown in "ball-and-stick" format. These formats are options selected using RasMol software. The dark parts of the ribbons represent domains of the cytokines and growth factors that are reported to be involved in binding to their receptors. For each structure, the accession code in the Protein Data Bank ("PDB") is indicated (see Laskowski, R.A., (2001) *Nucleic Acids Res 29*:221-222; Peitsch, M.C., (2002) *Bioinformatics 18*:934-938; Schein, C.H., (2002) *Curr Pharm Des 8*:2113-2129).

[0024] **Figure 1a** shows a model of interferon-*alpha*-2a (SEQ ID NO:1), in which the four lysine residues (Lys 31, Lys 121, Lys 131 and Lys 134) that are reported to be the primary sites of PEGylation in Roche's PEG-interferon product, PEGASYS®, are shown in "ball-and-stick" format (based on data of

Bailon, P., *et al.*, *supra*). The regions involved in binding to its receptors ("Binding Sites 1 and 2") are identified. All four of the lysine residues that are reported to be PEGylated in PEGASYS are in the region of Binding Site 1. (PDB code 1ITF)

[0025] **Figure 1b** shows a model of interferon-*alpha*-2b (SEQ ID NO:2), in which the residues that are reported to be the major sites of PEGylation in Schering-Plough's PEG-INTRON® (His 34, Lys 31, Lys 121, Tyr 129 and Lys 131) are shown in "ball-and-stick" format (based on data of Wylie, D.C., *et al., supra*). These amino acid residues are in the region of Binding Site 1.

[0026] **Figure 1c** shows a model of interferon-*alpha*-2b, in which the amino-terminal cysteine residue ("Cys 1"), a target of PEGylation according to the present invention, is shown in "ball-and-stick" format. Cys 1 is remote from Binding Sites 1 and 2.

[0027] **Figure 1d** shows the same model of interferon-*alpha*-2b as that shown in Figure 1c, to which a single strand of 20-kDa PEG has been attached at the N-terminal cysteine residue ("Cys 1"). The structure of PEG was generated using an adaptation of the program described by Lee, L.S., *et al.*, ((1999) *Bioconjug Chem 10*:973-981) and is rendered to the same scale as is the protein.

[0028] **Figure 2** shows a molecular model of human interferon-*beta*-1a (SEQ ID NO:3), in which several lysine residues that are within or adjacent to the receptor-binding domains are indicated (Lys 19, Lys 33, Lys 99 and Lys 134). In addition, the glycosylation site (Asn 80) and the N-terminal methionine residue ("Met 1") are shown in "ball-and-stick" format (based on data of Karpusas, M., *et al.*, (1997) *Proc Natl Acad Sci USA 94*:11813-11818; Karpusas, M., *et al.*, (1998) *Cell Mol Life Sci 54*:1203-1216; Runkel, L., *et al.*, (2000) *Biochemistry 39*:2538-2551). Met 1 is remote from Binding Sites 1 and 2, whereas several lysine residues are located within the receptor-binding domains. (PDB code 1AUI) The structure of interferon-*beta*-1b differs from that of interferon-*beta*-1a in lacking the N-terminal methionine

residue and carbohydrate moiety, as well as having a serine residue substituted for the unpaired cysteine residue (Cys 17).

[0029] **Figure 3** shows a molecular model of human granulocyte-macrophage colony-stimulating factor ("GM-CSF;" SEQ ID NO:5) in which three lysine residues (Lys 72, Lys 107 and Lys 111) that are within the receptor-binding domains, as well as the first amino acid residue near the amino terminus that is visualized in the crystal structure ("Arg 4"), are shown in "ball-and-stick" format (based on data of Rozwarski, D.A., *et al.*, (1996) *Proteins 26*:304-313). The amino-terminal region of GM-CSF is remote from Binding Sites 1 and 2. (PDB code 2GMF)

[0030] **Figure 4** shows a molecular model of human interleukin-2 ("IL-2;" SEQ ID 6), in which the amino acid residues that are reported to be involved with each of three receptors (*alpha*, *beta* and *gamma*) are represented in "ball-and-stick" format, as are several lysine residues that are within or close to the receptor-binding domains. The closest amino acid residue to the amino terminus that is visualized in the crystal structure is serine 6 ("Ser 6"), which is remote from the receptor-binding domains (based on data of Bamborough, P., *et al.*, (1994) *Structure 2*:839-851; Pettit, D.K., *et al.*, *supra*). (PDB code 3INK)

[0031] **Figure 5** shows a molecular model of human epidermal growth factor ("EGF;" SEQ ID NO:7) in "cartoon" format, except for the residues that are implicated in receptor binding and the two lysine residues (Lys 28 and Lys 48) that are adjacent to receptor-binding regions. The intra-chain disulfide bonds are shown as dashed lines. The closest amino acid residue to the amino terminus that is visualized in the crystal structure on which this model is based is cysteine 6 ("Cys 6") (based on data of Carpenter, G., *et al.*, (1990) *J Biol Chem 265*:7709-7712; Lu, H.-S., *et al.*, (2001) *J Biol Chem 276*:34913-34917). The flexible portion of the amino terminus of EGF (residues 1-5) that is not visualized in the crystal structure does not appear to be in a receptor-binding region. (PDB code 1JL9)

[0032] **Figure 6** shows a molecular model of basic fibroblast growth factor ("bFGF;" SEQ ID NO:8) in "cartoon" format in which the residues involved in binding to the receptors and to heparin are identified by presentation in "ball-and-stick" format (based on data of Schlessinger, J., *et al.*, (2000) *Mol Cell 6*:743-750). The first 12 amino acid residues from the amino terminus have not been implicated in receptor binding. (PDB code 1FQ9)

[0033] **Figure 7** shows a molecular model of insulin-like growth factor-1 ("IGF-1;" SEQ ID NO:9) in "cartoon" format, except for the residues involved in receptor binding (23-25 and 28-37), and glutamic acid residue 3 ("Glu 3"), which is the closest amino acid residue to the amino terminus that is visualized in the crystal structure. Two of the lysine residues are identified, one of which (Lys 27) is adjacent to the receptor-binding domain, and the other of which is remote from the receptor-binding domain (based on data of Brzozowski, A.M., *et al.*, (2002) *Biochemistry 41*:9389-9397). The amino terminus of IGF-1 is remote from the receptor-binding domains. (PDB code 1GZR)

[0034] **Figure 8** shows a molecular model of an interferon-*gamma* ("IFN-*gamma;*" SEQ ID NO:4), which is a homodimer. To clarify the interactions between the two polypeptide chains, one of the monomers ("Chain A") is shown in "ribbon" format and the other ("Chain B") is shown in "backbone" format. Lysine residues (shown in light "ball and stick" format) occur along the polypeptide chain, including the regions that are involved in the interface between the monomers or are adjacent to amino acid residues that are involved in receptor binding. The amino-terminal region of IFN-*gamma* is remote from the dimerization interface, but glutamine 1 (Gln 1) has been implicated in receptor binding. (Thiel, D.J., *et al.*, (2000) *Structure 8*:927-936; PDB code 1FG9)

[0035] **Figure 9** shows the fractionation of unPEGylated interferon-*alpha*-2b ("IFN"), monoPEGylated interferon-*alpha*-2b ("PEG_1-IFN") and diPEGylated interferon *alpha*-2b ("PEG_2-IFN") by cation-exchange chromatography of a reaction mixture containing IFN, 20-kDa mPEG-aldehyde and a reducing agent.

[0036] **Figure 10** shows size-exclusion chromatographic analysis of the reaction mixture fractionated as shown in Figure 9 and of selected fractions collected from the ion-exchange column for which results are shown in Figure 9.

[0037] **Figure 11** shows the fractionation by cation-exchange chromatography of a reaction mixture containing human IL-2, 20-kDa mPEG-aldehyde and a reducing agent. Under the indicated elution conditions, the residual unPEGylated IL-2 was not eluted from the column, unlike the results for interferon-*alpha*-2b shown in Figure 9.

[0038] **Figure 12** shows a size-exclusion chromatographic analysis of the reaction mixture fractionated as shown in Figure 11 and of selected fractions eluted from that column.

[0039] **Figure 13** shows electrophoretic analyses of a reaction mixture of PEGylated interleukin-2 ("PEG-IL-2") and of a fraction from the cation-exchange column for which the chromatogram is shown in Figure 11.

DETAILED DESCRIPTION OF THE INVENTION

[0040] Unless defined otherwise, all technical and scientific terms used herein have the same meanings as commonly understood by one of ordinary skill in the art to which this invention belongs. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and materials are described hereinafter.

Definitions

[0041] **About**: As used herein when referring to any numerical value, the term "about" means a value of ±10% of the stated value (*e.g.*, "about 50°C"

encompasses a range of temperatures from 45°C to 55°C, inclusive; similarly, "about 100 mM" encompasses a range of concentrations from 90 mM to 110 mM, inclusive).

[0042] **Amino Acid Residue**: As used herein, the term "amino acid residue" refers to a specific amino acid, usually dehydrated as a result of its involvement in two peptide bonds, in a polypeptide backbone or side chain, but also when the amino acid is involved in one peptide bond, as occurs at each end of a linear polypeptide chain. The amino acid residues are referred to by the three-letter codes or single-letter codes that are common in the art.

[0043] **Antagonist**: As used herein, the term "antagonist" refers to a compound, molecule, moiety or complex that reduces, substantially reduces or completely inhibits the biological and/or physiological effects of a given cytokine, chemokine, growth factor or polypeptide hormone on a cell, tissue or organism that are mediated through the receptors for the given cytokine, chemokine, growth factor or polypeptide hormone. Antagonists may carry out such effects in a variety of ways, including but not limited to competing with the agonist for binding site(s) or receptor(s) on the cell surface; interacting with the agonist in such a way as to reduce, substantially reduce or inhibit the ability of the agonist to bind to cell surface receptors; binding to and inducing a conformational change in cell surface receptors such that the receptors assume a structure to which the agonist can no longer bind (or can bind only with reduced or substantially reduced affinity and/or efficiency); inducing a physiological change (*e.g.*, increase in intracellular signaling complexes; increase in transcriptional inhibitors; reduction in cell surface ligand receptor expression; *etc.*) in cells, tissues or organisms such that the binding of the agonist, or the physiological signal induced by the agonist upon binding to the cell, is reduced, substantially reduced or completely inhibited; and other mechanisms by which antagonists may carry out their activities, that will be familiar to the ordinarily skilled artisan. As the ordinarily skilled artisan will understand, an antagonist may have a similar structure to the ligand that it

antagonizes (*e.g.*, the antagonist may be a mutein, variant, fragment or derivative of the agonist), or may have a wholly unrelated structure.

[0044] **Bioactive Component**: As used herein, the term "bioactive component" refers to a compound, molecule, moiety or complex that has a particular biological activity *in vivo*, *in vitro* or *ex vivo* upon a cell, tissue, organ or organism, and that is capable of being bound to one or more polyalkylene glycols to form the conjugates of the invention. Preferred bioactive components include, but are not limited to, proteins and polypeptides such as those that are described herein.

[0045] **Bound**: As used herein, the term "bound" refers to binding or attachment that may be covalent, *e.g.*, by chemically coupling, or non-covalent, *e.g.*, ionic interactions, hydrophobic interactions, hydrogen bonds, *etc.* Covalent bonds can be, for example, ester, ether, phosphoester, thioester, thioether, urethane, amide, amine, peptide, imide, hydrazone, hydrazide, carbon-sulfur bonds, carbon-phosphorus bonds, and the like. The term "bound" is broader than and includes terms such as "coupled," "conjugated" and "attached."

[0046] **Conjugate/conjugation**: As used herein, "conjugate" refers to the product of covalent attachment of a polymer, *e.g.*, PEG or PEO, to a bioactive component, *e.g.*, a protein or glycoprotein. "Conjugation" refers to the formation of a conjugate as defined in the previous sentence. Any method normally used by those skilled in the art of conjugation of polymers to biologically active materials can be used in the present invention.

[0047] **Coupled**: The term "coupled", as used herein, refers to attachment by covalent bonds or by strong non-covalent interactions, typically and preferably to attachment by covalent bonds. Any method normally used by those skilled in the art for the coupling of biologically active materials can be used in the present invention.

[0048] **Cytokine/Chemokine**: As used herein, the term "cytokine" is defined as a secreted regulatory protein that controls the survival, growth, differentiation, and/or effector function of cells, in endocrine, paracrine or

autocrine fashion (reviewed in Nicola, N.A., *supra;* Kossiakoff, A.A., *et al., supra*). Analogously, as used herein, the term "chemokine" is defined as a member of a family of structurally related glycoproteins with potent leukocyte activation and/or chemotactic activities (reviewed in Oppenheim, J.J., *et al., supra*). According to these definitions, cytokines and chemokines include interleukins, colony-stimulating factors, growth factors, and other peptide factors produced by a variety of cells, including but not limited to those specifically disclosed or exemplified herein. Like their close relatives, the polypeptide hormones and growth factors, cytokines and chemokines initiate their regulatory functions by binding to specific receptor proteins on the surface of their target cells.

[0049] **Disease, disorder, condition**: As used herein, the terms "disease" or "disorder" refer to any adverse condition of a human or animal including tumors, cancer, allergies, addiction, autoimmunity, infection, poisoning or impairment of optimal mental or bodily function. "Conditions" as used herein includes diseases and disorders but also refers to physiologic states. For example, fertility is a physiologic state but not a disease or disorder. Compositions of the invention suitable for preventing pregnancy by decreasing fertility would therefore be described as a treatment of a condition (fertility), but not a treatment of a disorder or disease. Other conditions are understood by those of ordinary skill in the art.

[0050] **Effective Amount**: As used herein, the term "effective amount" refers to an amount of a given conjugate or composition that is necessary or sufficient to realize a desired biologic effect. An effective amount of a given conjugate or composition of the present invention would be the amount that achieves this selected result, and such an amount can be determined as a matter of routine by a person skilled in the art, using assays that are known in the art and/or that are described herein, without the need for undue experimentation. For example, an effective amount for treating an immune system deficiency could be that amount necessary to cause activation of the immune system, resulting in the development of an antigen-specific immune

response upon exposure to an antigen. The term is also synonymous with "sufficient amount." The effective amount for any particular application can vary depending on such factors as the disease or condition being treated, the particular composition being administered, the route of administration, the size of the subject, and/or the severity of the disease or condition. One of ordinary skill in the art can determine empirically the effective amount of a particular conjugate or composition of the present invention without necessitating undue experimentation.

[0051] **One, a, or an**: When the terms "one," "a," or "an" are used in this disclosure, they mean "at least one" or "one or more," unless otherwise indicated.

[0052] **PEG**: As used herein, "PEG" includes all polymers of ethylene oxide, whether linear or branched or multi-armed and whether end-capped or hydroxyl terminated. "PEG" includes those polymers that are known in the art as poly(ethylene glycol), methoxypoly(ethylene glycol) or mPEG or poly(ethylene glycol)-monomethyl ether, alkoxypoly(ethylene glycol), poly(ethylene oxide) or PEO, α-methyl-ω-hydroxy-poly(oxy-1,2-ethanediyl) and polyoxirane, among other names that are used in the art for polymers of ethylene oxide.

[0053] **PEGylation, PEGylated and Mock PEGylated**: As used herein, "PEGylation" refers to any process for the covalent coupling of PEG to a bioactive target molecule, especially a receptor-binding protein. The conjugate produced thereby is referred to as being "PEGylated." As used herein, "Mock PEGylated" refers to the portion of the protein or other bioactive component in a PEGylation reaction mixture to which no PEG has become covalently attached. Nevertheless, the Mock PEGylated product may have been altered during the reaction or subsequent purification steps, *e.g.*, as a consequence of exposure to a reducing agent during PEGylation by reductive alkylation and/or by having one or more inhibitory agents, compounds, *etc.*, removed during the processing and/or purification steps.

[0054] **Polypeptide**: As used herein, the term "polypeptide" refers to a molecule composed of monomers (amino acids) linearly linked by amide bonds (also known as peptide bonds). It indicates a molecular chain of amino acids and does not refer to a specific length of the product. Thus, peptides, dipeptides, tripeptides, oligopeptides and proteins are included within the definition of polypeptide. This term is also intended to refer to the products of post-expression modifications of the polypeptide, for example, glycosylation, hyperglycosylation, acetylation, phosphorylation and the like. A polypeptide may be derived from a natural biological source or produced by recombinant technology, but is not necessarily translated from a designated nucleic acid sequence. It may be generated in any manner, including by chemical synthesis.

[0055] **Protein and glycoprotein**: As used herein, the term protein refers to a polypeptide generally of a size of above about 10 or more, 20 or more, 25 or more, 50 or more, 75 or more, 100 or more, 200 or more, 500 or more, 1,000 or more, or 2,000 or more amino acids. Proteins generally have a defined three-dimensional structure, although they do not necessarily have such structure, and are often referred to as folded, as opposed to peptides and polypeptides, which often do not possess a defined three-dimensional structure, but rather can adopt a large number of different conformations, and are referred to as unfolded. Peptides may, however, also have a defined three-dimensional structure. As used herein, the term glycoprotein refers to a protein coupled to at least one carbohydrate moiety that is attached to the protein *via* an oxygen-containing or a nitrogen-containing side chain of an amino acid residue, *e.g.*, a serine residue or an asparagine residue.

[0056] **Remote**: As used herein, the term "remote" (as in "remote N-terminal amino acid" or "remote glycosylation site") refers to a structure in which the location of one or more attachment sites for one or more polymers on a protein is/are distal to or spatially removed from one or more receptor-binding regions or domains of the protein, as assessed by molecular modeling. Conjugation of a polymer at such a remote attachment site (usually the N-terminal amino acid

(for receptor-binding proteins that are therefore referred to as "remote N-terminal" or "RN" receptor-binding proteins) or one or more carbohydrate moieties or glycosylation sites on a glycoprotein (for receptor-binding proteins that are therefore referred to as "remote glycosylation" or "RG" receptor-binding proteins)) does not cause substantial steric hindrance of the binding of the protein to its receptor(s). Hence, an amino-terminal amino acid or a glycosylation site on a cytokine, chemokine, growth factor or polypeptide hormone is said to be "located remotely from one or more receptor-binding domains" of the cytokine, chemokine, growth factor or polypeptide hormone when conjugation (*e.g.*, covalent attachment) of a water-soluble polymer to the amino-terminal amino acid or glycosylation site, respectively, does not interfere substantially with the ability of the cytokine, chemokine, growth factor or polypeptide hormone to bind to its receptor(s), particularly to cell-surface receptors. It is recognized, of course, that a given cytokine, chemokine, growth factor or polypeptide hormone may contain more than one receptor-binding domain. In such situations, an amino-terminal amino acid or glycosylation site of a cytokine, chemokine, growth factor or polypeptide hormone can be located remotely from one such domain or from more than one of such domains, and still be considered to be "located remotely from one or more receptor-binding domains," so long as conjugation of the amino-terminal amino acid or glycosylation site does not interfere substantially with the binding of the cytokine, chemokine, growth factor or polypeptide hormone to its receptor(s) *via* one or more of the receptor-binding domains. Whether or not the conjugation interferes substantially with the ability of a protein to bind to its receptor(s) can be readily determined using art-known assays of ligand-receptor binding that will be familiar to the ordinarily skilled artisan.

[0057] Methods of assessing ligand-receptor binding include, without limitation, competitive binding assays, radioreceptor binding assays, cell-based assays, surface plasmon resonance measurements, dynamic light scattering and ultracentrifugation.

[0058] As shown in Figure 1d of this specification, PEG is a highly extended and flexible polymer that occupies a large volume in solution relative to a protein of similar molecular weight. Although the amino acid residue to which the PEG is attached may be remote from one or more receptor-binding sites, portions of the polymer could, nevertheless, interfere, to some extent, with receptor binding. The probability of such interference increases with the molecular weight and hence the volume occupied by the polymer in solution. Finally, PEGylation that is remote from the receptor-binding regions will interfere less with receptor binding than random PEGylation.

[0059] **Substantially, substantial**: As used herein, conjugation of a protein is said not to interfere "substantially" with the ability of the protein to bind to its receptor(s) if the rate and/or amount of binding of a conjugated protein to a receptor is not less than about 40%, about 50%, about 60%, about 65%, about 70%, about 75%, about 80%, about 85%, about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, about 99% or about 100% or more, of the binding rate and/or amount of the corresponding cytokine, chemokine, growth factor or polypeptide hormone that has not been conjugated.

[0060] **Treatment**: As used herein, the terms "treatment," "treat," "treated" or "treating" refer to prophylaxis and/or therapy. When used with respect to an infectious disease, for example, the term may refer to a prophylactic treatment that increases the resistance of a subject to infection with a pathogen or, in other words, decreases the likelihood that the subject will become infected with the pathogen or will show signs of illness attributable to the infection, as well as a treatment after the subject has become infected in order to fight the infection, *e.g.*, to reduce or eliminate the infection or to prevent it from becoming worse.

Overview

[0061] The present invention provides methods for the synthesis of polymer conjugates of receptor-binding proteins that retain unexpectedly high receptor-

binding activity relative to polymer conjugates of the same receptor-binding protein in which one or more polymers is/are attached randomly. Through the use of x-ray crystallographic and nuclear magnetic resonance-based structural analyses, mutational analysis and molecular modeling software, the present inventors have identified target sites for PEGylation of cytokines, chemokines, growth factors and polypeptide hormones that are involved or are not involved in binding to their receptors. As a class of proteins, these cytokines, chemokines, growth factors and polypeptide hormone agonists and antagonists are referred to herein as receptor-binding proteins. By selection of a synthetic strategy that targets polymer attachment to the region(s) of receptor-binding proteins that are not involved in receptor interactions, certain undesirable steric hindrances are avoided and the resultant polymer conjugates retain unusually high potency. Those receptor-binding proteins that have an amino-terminal residue that is remote from one or more of their receptor-binding regions or domains are defined herein as "remote N-terminal" or "RN" receptor-binding proteins; they include all cytokines, chemokines, growth factors and polypeptide hormones or antagonists thereof that have their amino-terminal amino acid located remotely from the receptor-binding site or sites of the protein.

[0062] In another embodiment of the invention, conjugates are produced comprising one or more synthetic polymers (*e.g.*, one or more poly(ethylene glycols)) covalently coupled to cytokines, chemokines, growth factors and polypeptide hormones that have natural glycosylation sites that are remote from one or more of their receptor-binding regions or domains. According to this aspect of the invention, the bioactive components (*e.g.*, proteins) of the conjugates will display well-preserved receptor-binding activities when synthetic polymers are coupled in the region of the glycosylation site(s). This subset of receptor-binding proteins is referred to herein as "RG" receptor-binding proteins. When a hydrophilic or amphipathic polymer is selectively coupled at or near such a "remote glycosylation" site, especially when the target protein is a non-glycosylated form of a protein that is naturally

glycosylated, the polymer can mimic the favorable effects of the naturally occurring carbohydrate, *e.g.*, on aggregation, stability and/or solubility, and hence its attachment is referred to herein as "pseudoglycosylation." Hence, the present invention provides methods for the synthesis of conjugates in which the site-selective coupling of a synthetic polymer effectively replaces the naturally occurring carbohydrate moieties. The resultant pseudo-glycosylation contributes to improved solubility, decreased aggregation and retarded clearance from the bloodstream, compared to other nonglycosylated forms of the protein. This approach therefore is particularly advantageous for preparing conjugates and compositions of proteins that are produced by recombinant DNA techniques in prokaryotic host cells (*e.g.*, bacteria such as *Escherichia coli*), since prokaryotic organisms generally do not glycosylate proteins that they express. Analogously, selective PEGylation of the carbohydrate moiety of a glycoprotein can result in "pseudohyper-glycosylation" of the glycoprotein. This process was described, for example, by C. Bona *et al.*, in PCT Publication No. WO 96/40731, the disclosure of which is incorporated herein by reference in its entirety. This approach therefore is particularly advantageous for preparing conjugates and compositions of proteins that are produced by recombinant DNA techniques in eukaryotic host cells (*e.g.*, in yeasts, plant cells and animal cells (including mammalian and insect cells), since eukaryotic organisms generally do glycosylate proteins that they express, if those proteins include naturally occurring glycosylation signals or glycosylation signals introduced by recombinant DNA technology. Such pseudoglycosylated and pseudohyper-glycosylated RG receptor-binding proteins are within the scope of the present invention.

[0063] The invention thus also encompasses polymer conjugates of "RN" receptor-binding proteins that retain substantial, nearly complete or essentially complete receptor-binding activity and pseudoglycosylated or pseudo-hyperglycosylated "RG" receptor-binding proteins that retain substantial, nearly complete or essentially complete receptor-binding activity. As used

herein, a cytokine, chemokine, growth factor or polypeptide hormone is said to "retain substantial, nearly complete or essentially complete receptor-binding activity" when conjugated with one or more water-soluble polymers according to the present invention, if the conjugation of the cytokine, chemokine, growth factor or polypeptide hormone does not interfere substantially with the ability of the protein to bind to its receptor(s), *i.e.*, if the rate and/or amount of binding of the conjugated protein to its corresponding receptor(s) is not less than about 40%, about 50%, about 60%, about 65%, about 70%, about 75%, about 80%, about 85%, about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, about 99% or about 100% or more, of the binding rate and/or amount of an unconjugated form of the corresponding protein. Also included within the scope of the present invention are polymer conjugates of those receptor-binding proteins that are classified as both "RN" and "RG" receptor-binding proteins. Two examples of the latter proteins are interferon *beta* (particularly interferon-*beta*-1b) and IL-2.

[0064] In additional embodiments, the invention provides methods for the synthesis of polymer conjugates of receptor-binding proteins that retain unexpectedly high receptor-binding activity relative to polymer conjugates of the same receptor-binding protein in which one or more polymers is/are attached randomly. The invention also provides conjugates produced by such methods, and compositions comprising one or more of these conjugates of the invention that may further comprise one or more additional components or reagents, such as one or more buffer salts, one or more carbohydrate excipients, one or more carrier proteins, one or more enzymes, one or more detergents, one or more nucleic acid molecules, one or more polymers such as unconjugated PEG or polyalkylene glycol, and the like. The invention also provides kits comprising the conjugates and/or compositions of the invention.

[0065] The invention also provides pharmaceutical or veterinary compositions comprising the conjugates of the invention and at least one excipient or carrier that is acceptable for pharmaceutical or veterinary use. The invention also

provides methods of treating or preventing a variety of physical disorders using such compositions, comprising administering an effective amount of one or more of the conjugates or compositions of the present invention to an animal suffering from or predisposed to a physical disorder or condition.

[0066] Further, the invention provides stabilized receptor-binding proteins and methods for their production for use in industrial cell culture, whereby unexpectedly high potencies are obtained as a result of the combined effects of substantial retention of bioactivity and increased duration of action in industrial use. The unusually high potencies of the conjugates of the present invention may be reflected in unusually high biomass production, unusually high levels of expression of recombinant proteins and other improvements in efficiencies of bioprocessing.

Methods

[0067] The present inventors have discovered that targeting of polymers to the amino-terminal amino acid of an "RN" receptor-binding protein or to the vicinity of the glycosylation site of an "RG" receptor-binding protein assures that the polymer is attached at a site that is remote from one or more of the receptor-binding regions or domains of the protein, thereby minimizing steric hindrance of receptor interactions by the attached polymer molecules. Consequently, a higher percentage of the receptor-binding activity can be preserved by conjugating proteins according to the methods of the present invention than would occur if the polymer were attached within or proximal to a portion of the molecule that is involved in binding to its receptor(s). This principle, which can result in unexpectedly high retention of receptor binding activity, can be demonstrated for receptor-binding proteins that are selected from among basic fibroblast growth factor ("bFGF" or "FGF-2"), epidermal growth factor ("EGF"), insulin-like growth factor-1 ("IGF-1"), interferon-*alpha* ("IFN-*alpha*"), interferon-*beta* ("IFN-*beta*" (including IFN-*beta*-1b)), granulocyte-macrophage-colony stimulating factor ("GM-CSF"), monocyte colony stimulating factor ("M-CSF"), Flt3 ligand, stem cell factor ("SCF"),

interleukins 2, 3, 4, 6, 10, 12, 13 and 15, tumor necrosis factor-*alpha* ("TNF-*alpha*"), tumor necrosis factor-*beta* ("TNF-*beta*"), transforming growth factor-*alpha* ("TGF-*alpha*"), transforming growth factor-*beta* ("TGF-*beta*"), keratinocyte growth factor ("KGF"), human growth hormone ("hGH"), prolactin, placental lactogenic hormone, ciliary neurotrophic factor ("CNTF"), leptin and structural analogs of these receptor-binding-proteins that mimic the actions of these proteins or that are receptor-binding antagonists thereof. In contrast, the selective attachment of a large polymer to the amino terminus of IFN-*gamma* is not predicted to preserve most of the activity of this cytokine, since such coupling is expected to interfere with binding of the active dimer to its receptors (based on data of Walter, M.R., *et al.*, (1995) *Nature 376*:230-235 and Thiel, D.J., *et al.*, *supra*).

[0068] In a related such embodiment of the invention, polymers are coupled to the amino-terminal residue of muteins of receptor-binding proteins that function as competitive antagonists of the natural protein by binding to one or more of the same receptor(s) without initiating signal transduction. Examples are polymer conjugates of an hGH antagonist that contains the point mutation G120R (Sundström, M., *et al.*, (1996) *J Biol Chem 271*:32197-32203) and an antagonist of prolactin that contains the point mutation G129R (Goffin, V., *et al.*, (1997) *J Mammary Gland Biol Neoplasia 2*:7-17; Chen, W.Y., *et al.*, (1999) *Clin Cancer Res 5*:3583-3593; Chen, W.Y., PCT Publication No. WO 99/58142 A1). Other antagonists of receptor-binding proteins can be produced by selective point mutations, truncations or deletions (*see e.g.*, Tchelet, A., *et al.*, (1997) *Mol Cell Endocrinol 130*:141-152; Peterson, F.C., (1998) *Identification of Motifs Associated with the Lactogenic and Somatotropic Actions of Human Growth Hormone*, Ph.D. Dissertation, Ohio State University, UMI # 9822357).

[0069] In another embodiment of the invention, for "RG" receptor-binding proteins, the methods of the present invention result in the attachment of one or more synthetic polymers in proximity to the natural site of attachment of carbohydrate moieties of those receptor-binding proteins that are

glycoproteins. This results in "pseudoglycosylation" of these receptor-binding proteins (for example, when they have been expressed by recombinant DNA techniques in *E. coli* or other prokaryotic cells that do not perform post-translational glycosylation) or results in "pseudohyperglycosylation" of their glycoprotein forms (for example, for naturally produced glycoproteins or for glycoproteins produced by eukaryotic host cells (*e.g.*, yeasts, plant cells and animal cells (including mammalian and insect cells), that do perform post-translational glycosylation). Examples are polymer conjugates of interferons *alpha* and *beta*, as well as of erythropoietin ("Epo") and interleukin-2. The attachment of synthetic polymers at or near the sites of natural glycosylation can be performed by any method that is known in the art, including the mutational method of R.J. Goodson *et al.*, ((1990) *Biotechnology 8*:343-346) and the method of R.S. Larson *et al.*, ((2001) *Bioconjug Chem 12*:861-869), which involves prior oxidation of the carbohydrate; the disclosures of these references are incorporated herein by reference in their entireties.

[0070] Amino-terminal modification of certain proteins has been disclosed previously (*see, e.g.* Dixon, H.B.F., (1984) *J Protein Chem 3*:99-108). For example, N-terminal modification of proteins has been reported to stabilize certain proteins against the action of aminopeptidases (Guerra, P.I., *et al.*, (1998) *Pharm Res 15*:1822-1827), to improve the solubility of the protein (Hinds, K., *et al.*, (2000) *Bioconjug Chem 11*:195-201), to decrease the charge on the N-terminal amino group, or to improve the homogeneity of the resulting conjugates (Kinstler, O., *et al.*, European Patent Publication No. EP 0 822 199 A2; Kinstler, O., *et al.*, (2002) *Adv Drug Deliv Rev 54*:477-485), among others. An alternative method for coupling polymers to the *alpha* amino group of an N-terminal cysteine or histidine residue, by an adaptation of a procedure known in the art as "native chemical ligation," has been disclosed (Roberts, M.J., *et al.*, PCT Publication No. WO 03/031581 A2 and U.S. Patent Application Publication No. 2003/0105224). However, the existence of the "RN" and "RG" subclasses of receptor-binding proteins, generally applicable methods for selecting members of those classes, and the

preparation and use of polymer conjugates of such receptor-binding proteins as a way to preserve unexpectedly high functional activity of "RN" receptor-binding proteins, have not been recognized or described previously.

[0071] Hence, there is an advantage to determining whether or not a given cytokine, chemokine, growth factor or polypeptide hormone has an N-terminus and/or glycosylation site(s) that is/are remote from the receptor-binding site(s) of the ligand. The ability to predict whether a given cytokine, chemokine, growth factor or polypeptide is an "RN" or an "RG" ligand, prior to conjugation of the ligand with a polymer, substantially decreases the experimentation required to produce polymer-ligand conjugates (*e.g.*, cytokines, chemokines, growth factors, polypeptide hormones or antagonists thereof conjugated with polymers, *e.g.*, PEGs) in which the antigenicity and immunogenicity of the conjugate is reduced relative to the antigenicity/immunogenicity of the unconjugated ligand, while not substantially decreasing the receptor-binding and physiological activities of the conjugated ligand.

[0072] Accordingly, in additional embodiments, the present invention provides methods for identifying and selecting receptor-binding protein ligands (*e.g.*, cytokines, chemokines, growth factors, polypeptide hormones and antagonists thereof) that have an N-terminus and/or glycosylation site(s) that are remote from the receptor-binding sites of the protein ligands (*i.e.*, methods for identifying and selecting for "RN" or "RG" proteins). In certain such embodiments of the invention, the optimum location for conjugation of one or more polymers (*e.g.*, one or more PEGs) can be determined using molecular modeling, *e.g.*, by viewing the 3-dimensional structure of the protein (cytokine, chemokine, growth factor, polypeptide hormone or antagonist thereof) using molecular modeling software to predict the location(s) at which one or more polymers can be attached to the protein without a substantial loss in biological or receptor-binding activity of the protein (see also Schein, C.H., *supra*). An analogous approach has been demonstrated, for example, for conjugation of PEG to G-CSF in an attempt to

improve its resistance to proteolytic digestion (*see* published U.S. Application No. 2001/0016191 A1 of T.D. Osslund, the disclosure of which is incorporated by reference herein in its entirety). Suitable molecular modeling software for use in the present invention, such as RASMOL (Sayle, R.A., *et al.*, *supra*) and other programs used in generating the database of macromolecular structures deposited at the Protein Data Bank (PDB; *see* Laskowski, R.A., *supra*), is well-known in the art and will be familiar to those of ordinary skill in the art. Using such molecular modeling software, the three-dimensional structure of a polypeptide, *e.g.*, a cytokine, chemokine, growth factor, polypeptide hormone, or antagonist thereof, can be predicted or determined with a high degree of confidence, based on crystallographic analyses of the ligands and their receptors. In this way, one of ordinary skill can readily determine which ligands are "RN" or "RG" ligands that are suitable for use in accordance with the present invention.

[0073] To practice the present invention, one convenient route for covalently coupling a water-soluble polymer to the *alpha* amino group of the N-terminal amino acid residue of a protein is by reductive alkylation of Schiff's bases formed with polymers bearing a single aldehyde group, *e.g.* as claimed by G.P. Royer (U.S. Patent No. 4,002,531), but not as claimed by J.M. Harris *et al.*, (U.S. Patent No. 5,252,714), since the latter inventors claim only polymers derivatized at both ends with aldehyde groups, which are cross linking agents and are therefore ill-suited to the synthesis of long-acting receptor-binding proteins that retain substantial receptor-binding activity.

[0074] Directing the reductive alkylation of Schiff's bases of PEG-monoaldehydes toward the *alpha* amino group of the N-terminal amino acid of a receptor-binding protein and away from the *epsilon* amino groups of its lysine residues can be accomplished by a variety of methods, based on the disclosures in J.T. Edsall in Chapters 4 and 5 of *Proteins Amino Acids and Peptides as Ions and Dipolar Ions* ((1943), pp. 75-115 and pp. 116-139, Reinhold Publishing Corporation, New York), the disclosure of which is incorporated herein by reference in its entirety. The acidic dissociation

constant ("pK_a") of an *alpha* amino group of an N-terminal amino acid of a polypeptide is expected to be below 7.6, whereas the pK_a values of the *epsilon* amino groups of lysine residues in polypeptides are expected to be approximately 9.5. Edsall ((1943, *supra*) clearly stated that aldehydes will combine with the amino group of an amino acid "only on the alkaline side of its isoelectric point."

[0075] Hence, based on the present disclosure and information that is readily available in the art, the ordinarily skilled artisan will recognize that (1) the selective reaction of aldehydes with the *alpha* amino group of a protein will be favored by a range of pH that is below 9.5 (approximately the pK_a of the *epsilon* amino groups in the protein); (2) the rate of reaction of aldehydes with *epsilon* amino groups will decrease if the pH of the reaction is lowered toward 7.6 (approximately the pK_a of the *alpha* amino group of the protein); (3) the rate of reaction of aldehydes with the *alpha* amino group will decrease less than that of the *epsilon* amino groups as the reaction pH is lowered toward 7.6, and (4) the selectivity for the reaction of an aldehyde with the *alpha* amino group will be improved somewhat by lowering the pH toward 6.6. Since the latter value is approximately one pH unit below the pK_a of the *alpha* amino group and three pH units below the pK_a of the epsilon amino groups, approximately 10% of the *alpha* amino groups and approximately 0.1% of the *epsilon* amino groups will be in their reactive, unprotonated state. Thus at pH 6.6, the fraction of unprotonated *alpha* amino groups is 100-fold higher than the fraction of unprotonated *epsilon* amino groups. Therefore, very little increase in selectivity will be obtained by lowering the pH of the reaction further, *e.g.*, to 5.6, where, theoretically, 1% of the *alpha* amino groups and 0.01% of the *epsilon* amino groups would be in their reactive, unprotonated state. Thus, in certain embodiments of the invention, protein ligands (particularly "RN" or "RG" ligands, including cytokines, chemokines, growth factors, polypeptide hormones and antagonists thereof) are conjugated with one or more polymers by forming a mixture between the ligand(s) and the one

or more polymers at a pH of about 5.6 to about 7.6; at a pH of about 5.6 to about 7.0; at a pH of about 6.0 to about 7.0; at a pH of about 6.5 to about 7.0; at a pH of about 6.6 to about 7.6; at a pH of about 6.6 to about 7.0; or at a pH of about 6.6. The present methods thus differ significantly from those known in the art, in which coupling of polymers to *alpha* amino groups on the N-terminal amino acid residues of ligands is carried out at a pH of about 5 (Kinstler, O., *et al.*, (2002) *Adv Drug Deliv Rev 54*:477-485; European Patent Publication No. EP 0 822 199 A2; U.S. Patent Nos. 5,824,784 and 5,985,265; Roberts, M.J., *et al.*, (2002) *supra*; Delgado, C., *et al.*, U.S. Application Publication No. 2002/0127244 A1), while coupling of polymers to *epsilon* amino groups of lysine residues in the ligand polypeptide backbone is carried out at a pH of 8.0 (Kinstler, O., *et al.*, EP 0 822 199 A2; U.S. Patent Nos. 5,824,784 and 5,985,265). In the same way, the present methods also are significantly distinct from enzymatic methods that have been used for coupling alkylamine derivatives of poly(ethylene glycol) to certain proteins using transglutaminase, which is carried out at a pH of 7.5 (Sato, H., (2002), *Adv Drug Deliv Rev 54*:487-504).

[0076] Reduction of the resultant Schiff's bases with mild reducing agents, such as sodium cyanoborohydride or pyridine borane (Cabacungan, J.C., *et al.*, (1982) *Anal Biochem 124*:272-278), forms secondary amine bonds that preserve the positive charge of the N-terminal *alpha* amino group of the protein at physiological pH. Such bonds that retain the same charge as the native protein are more likely to preserve its biological activity than alternative linkage chemistries that neutralize the charge, *e.g.*, by the formation of amide bonds (Burg, J., *et al.*, PCT Publication No. WO 02/49673 A2; Kinstler, O., *et al.*, European Patent Application No. EP 0 822 199 A2; Kinstler, O.B., *et al.*, (1996) *Pharm Res*, *13*:996-1002; Kita, Y., *et al.*, *supra*) or urethane bonds (Gilbert, C.W., *et al.*, U.S. Patent No. 6,042,822; Grace, M., *et al.*, (2001) *J Interferon Cytokine Res 21*:1103-1115; Youngster, S., *et al.*, (2002) *Curr Pharm Des 8*:2139-2157).

[0077] Alternative approaches to selective coupling of polymers to N-terminal amino acid residues are known to those skilled in the art. Included are methods for coupling hydrazide, hydrazine, semicarbazide or other amine-containing polymers to N-terminal serine or threonine residues that have been oxidatively cleaved to aldehydes with periodate (Dixon, H.B.F., *supra*; Geoghegan, K.F., U.S. Patent No. 5,362,852; Gaertner, H.F., *et al.*, (1996) *Bioconjug Chem* 7:38-44; Drummond, R.J., *et al.*, U.S. Patent No. 6,423,685).

Suitable Polymers

[0078] In certain embodiments of the invention, it is desirable to minimize the formation of intramolecular and intermolecular cross-links by polymers such as PEG during the reaction in which the polymer is coupled to the bioactive component to produce the conjugates of the invention. This can be accomplished by using polymers that are activated at only one end (referred to herein as "monofunctionally activated PEGs" or "monofunctionally activated PAGs") or polymer preparations in which the percentage of bifunctionally activated (referred to in the case of linear PEGs as "*bis*-activated PEG diols") or multi-functionally activated polymers is less than about 30%, or more preferably less than about 10% or most preferably less than about 2% (w/w). The use of activated polymers that are entirely or nearly entirely monofunctional can minimize the formation of all of the following: intramolecular cross links within individual protein molecules, "dumbbell" structures, in which one strand of polymer connects two protein molecules, and larger aggregates or gels.

[0079] Activated forms of polymers that are suitable for use in the methods and compositions of this invention can include any linear or branched, monofunctionally activated forms of polymers that are known in the art. For example, included are those with molecular weights (excluding the mass of the activating group) in the range of about 1 kDa to about 100 kDa. Suitable ranges of molecular weights include but are not limited to about 5 kDa to about 30 kDa; about 10 kDa to about 20 kDa; about 18 kDa to about 60 kDa;

about 12 kDa to about 30 kDa, about 5 kDa, about 10 kDa, about 20 kDa or about 30 kDa. In the case of linear PEGs, molecular weights of about 10 kDa, about 20 kDa or about 30 kDa correspond to degrees of polymerization (*n*) of about 230, about 450 or about 680 monomeric units of ethylene oxide, respectively. For use *in vitro*, suitable ranges of molecular weights of activated polymers include about 1 kDa to about 5 kDa. It should be noted that long before the existence of the "RN" and "RG" classes of receptor-binding proteins was recognized, the advantages of coupling therapeutic proteins to polymers having relatively high molecular weights (*i.e.*, greater than about 20-30 kDa) were first observed (Saifer, M., *et al.*, PCT Publication No. WO 89/01033 A1, published Feb. 9, 1989, which is incorporated herein by reference in its entirety).

[0080] In other embodiments of the invention, conjugates of receptor-binding proteins with unusually high percentages of retained bioactivity can be prepared for use *in vitro, e.g.,* in cell culture, by coupling monofunctionally activated polymers of about 1 kDa, about 2 kDa or about 5 kDa, according to the methods of this invention. For such *in vitro* applications, this lower range of molecular weights may be preferred.

[0081] Optionally, a linear polymer can have a reactive group at one end or both ends, thereby creating a "reactive polymer." In certain embodiments of this invention, it can be desirable to use the N-hydroxysuccinimidyl ester of the monopropionic acid derivative of PEG, as disclosed in J.M. Harris *et al.*, U.S. Patent No. 5,672,662, which is incorporated herein fully by reference, or other N-hydroxysuccinimide-activated PEG-monocarboxylic acids. In certain other embodiments, it can be desirable to use either the monosuccinimidyl carbonate derivatives of PEG ("SC-PEG"), as described in M. Saifer *et al.*, U.S. Patent Nos. 5,006,333; 5,080,891; 5,283,317 and 5,468,478, or the mono-*p*-nitrophenyl carbonate derivative of PEG, as disclosed in S.J. Kelly *et al.*, *supra*; in L.D. Williams *et al.* PCT Publication No. WO 00/07629 A2 and A3; L.D. Williams *et al.*, U.S. Patent No. 6,576,235 and in M.R. Sherman *et al.*, PCT Publication No. WO 01/59078 A2. Moreover, other types of reactive

groups can be used to synthesize polymer conjugates of proteins. These derivatives include, but are not limited to, monoaldehyde derivatives of PEGs (Royer, G.P., U.S. Patent No. 4,002,531; Harris, J.M. *et al.*, U.S. Patent No. 5,252,714), monoamine, mono-tribromophenyl carbonate, monocarbonyl-imidazole, mono-trichlorophenyl carbonate, mono-trifluorophenyl carbonate, monohydrazide, monosemicarbazide, monocarbazate, monothiosemicarbazide, monoiodoacetamide, monomaleimide, mono-orthopyridyl disulfide, mono-oxime, mono-phenylglyoxal, mono-thiazolidine-2-thione, monothioester, monothiol, monotriazine and monovinylsulfone derivatives of PEGs. In additional embodiments, cytokines, chemokines, growth factors, polypeptide hormones and antagonists thereof can be coupled to one or more polymers as described in commonly owned, co-pending U.S. Patent Application No. 10/669,597, the disclosure of which is incorporated herein by reference in its entirety.

Bioactive Components

[0082] As noted above, the conjugates of the invention comprise one PAG or PAO, and particularly one strand of PEG, covalently attached to one or more bioactive components. Bioactive components to which one or more polymers (or strands thereof) has/have been covalently attached are referred to herein variously and equivalently as "conjugated bioactive components" or "modified bioactive components." These terms are to be distinguished herein from "unconjugated bioactive components," "initial bioactive components" or "unmodified bioactive components," all of which terms refer to bioactive components that have not had polymers covalently attached thereto. It is to be understood, however, that an "unconjugated," "unmodified" or "initial" bioactive component may contain other, non-polymer conjugations or modifications when compared to a wild-type or native molecule, and would still be considered to be "unconjugated," "unmodified" or "initial" in accordance with the present invention, since the bioactive component would be "unconjugated," "unmodified" or "initial" with respect to the attachment of

polymers, as is the case for bioactive components that are referred to herein as "Mock PEGylated."

[0083] The term "stabilizing" a bioactive component (or "methods of stabilization" or "stabilized bioactive component") indicates that a bioactive component has been stabilized according to the methods of this invention (*i.e.*, a bioactive component to which a polymer has been covalently attached according to the methods of the invention). Such stabilized bioactive components will exhibit certain altered biochemical and biophysical characteristics when compared to a bioactive component that has not been stabilized (*i.e.*, a bioactive component to which a polymer has not been covalently attached). Included among such altered biochemical and biophysical parameters, particularly for receptor-binding proteins, may be decreased susceptibility to proteolytic degradation and particularly the maintenance of the activity of a receptor-binding protein during incubation under certain harsh environmental or experimental conditions. In certain embodiments of the invention, the altered biochemical and biophysical parameters may include, for example, an increased half-life in the circulation *in vivo*, increased bioavailability, increased duration of action *in vitro*, and the like.

[0084] Any receptor-binding protein (typically a cytokine, chemokine, growth factor or polypeptide hormone) having biological (*i.e.*, physiological, biochemical or pharmaceutical) activity associated with portions of the molecule that are remote from its amino terminus or from a naturally occurring or mutationally-introduced glycosylation site can be suitably used as an initial component in the present invention. Such bioactive components include, but are not limited to, peptides, polypeptides, proteins and the like. Bioactive components also include fragments, muteins and derivatives of such peptides, polypeptides, proteins and the like, particularly such fragments, muteins and derivatives having biological (*i.e.*, physiological, biochemical or pharmaceutical) activity.

[0085] Suitable peptides, polypeptides and proteins, glycoproteins and the like that are useful as bioactive components in the present invention include any peptide, polypeptide or protein, *etc.*, having one or more than one available amino group, thiol group or other group that is remote from the receptor-binding region or regions of the bioactive component and to which polymers can be selectively attached. Such peptides, polypeptides, proteins, glycoproteins and the like include cytokines, chemokines, growth factors and polypeptide hormones, which may have any of a variety of structures (Nicola, N.A., *supra*; Schein, C.H., *supra*).

[0086] For example, suitable peptides, polypeptides and proteins of interest include, but are not limited to the class of cytokines having structures comprising four α-helical bundles (both long-chain and short-chain subclasses) (for review, *see* Schein, C.H., *supra*). A variety of such four-helical bundle proteins are suitable for use in the present invention, including but not limited to interleukins, *e.g.*, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-11, IL-12 (p35 subunit), IL-13, IL-15 and IL-17; colony-stimulating factors, *e.g.*, macrophage colony-stimulating factor (M-CSF) and granulocyte-macrophage colony-stimulating factor (GM-CSF; Rozwarski, D.A., *et al.*, (1996) *Proteins 26*:304-313); interferons, *e.g.*, IFN-α, IFN-β (including IFN-β-1b) and consensus IFN; leukemia inhibitory factor (LIF); erythropoietin (Epo); thrombopoietin (Tpo); megakaryocyte growth and development factor (MGDF); stem cell factor (SCF), also known in the art as Steel Factor (Morrissey, P.J., *et al.*, (1994) *Cell Immunol 157*:118-131; McNiece, I.K., *et al.*, (1995) *J Leukoc Biol 58*:14-22); oncostatin M (OSM); phospholipase-activating protein (PLAP); neurotrophic factors; and peptide mimetics thereof. Although prolactin and growth hormone are classical hormones, which circulate widely in the body, unlike the cytokines, which are usually produced near their target cells, prolactin and growth hormone belong to the same structural class as the cytokines with four α-helical bundles (Nicola, N.A., *supra*; Goffin, V., *et al.*, *supra*) and they are similarly suitable targets for polymer coupling and for production of the present conjugates in accordance

with the present invention. Analogues, muteins, antagonists, variants and derivatives of these peptides, polypeptides and proteins are also suitable for use in, and are therefore encompassed by, the present invention.

[0087] Receptor-binding proteins of the long chain β-sheet or β-barrel structural classes (for review, *see* Schein, C.H., *supra*) are also suitable for use in preparing the conjugates and compositions of the present invention. These include, but are not limited to: the tumor necrosis factor family of cytokines, *e.g.,* TNF-α, TNF-β and Fas ligands, which display β-jelly roll structures; the IL-1 (including IL-1α and IL-1β) and FGF (including basic fibroblast growth factor (bFGF), acidic FGF, FGF-4 and keratinocyte growth factor (KGF; FGF-7)) families, which show a *beta*-trefoil fold (Schein, C.H., *et al.*, *supra*; Schlessinger, J., *et al.*, *supra*); IL-12; IL-16; epidermal growth factor (EGF; Lu, H.-S., *et al.*, *supra*); and the platelet-derived growth factors (PDGFs), transforming growth factors (including transforming growth factor-α and transforming growth factor-β (TGF-β)) and nerve growth factors, which adopt cystine-knot structures. Analogues, muteins, antagonists, variants and derivatives of these peptides, polypeptides and proteins are also suitable for us in, and are therefore encompassed by, the present invention.

[0088] An additional structural class of proteins that are advantageously used in the conjugates and compositions of the present invention is that of the disulfide-rich mixed α/β cytokines, chemokines and growth factors (for review, *see* Schein, C.H., *supra*), including but not limited to: the EGF family, which has a *beta*-meander structure; IL-8; RANTES; neutrophil activating peptide-2 (NAP-2); stromal cell-derived factor-1α (SDF-1α); the monocyte chemoattractant proteins (MCP-1, MCP-2 and MCP-3); the eotaxins (*e.g.*, eotaxin-1, eotaxin-2 and eotaxin-3); myeloid progenitor inhibitory factor-1 (MPIF-1); neurotactin, macrophage migration inhibitory factor (MIF); growth-related oncogene/melanoma growth stimulatory activity (GRO-α/MGSA); somatomedins; and insulin and the insulin-like growth factors (*e.g.*, IGF-1 and IGF-2). A related structural class of proteins of use in the conjugates and compositions of the present invention is cytokines with mosaic structures,

which includes growth factors such as IL-12 and hepatocyte growth factor (Nicola, N.A., *supra*). Analogues, muteins, antagonists, variants and derivatives of these peptides, polypeptides and proteins are also suitable for us in, and are therefore encompassed by, the present invention.

[0089] Other proteins of interest include, but are not limited to: growth hormones (particularly human growth hormone (hGH; *see* Tchelet, A., *et al.*, *supra*) and antagonists thereof (*see, e.g.*, Sundström, M., *et al.*, *supra*), prolactin and antagonists thereof, chorionic gonadotropin, follicle-stimulating hormone, thyroid-stimulating hormone, pigmentary hormones, keratinocyte growth factor, hypothalamic releasing factors, antidiuretic hormones and receptor-binding antagonists of cytokines, chemokines, growth factors and polypeptide hormones of all of the above structural classes. Many such proteins exist in both glycosylated and non-glycosylated forms. The non-glycosylated forms may result from their production using recombinant DNA techniques in prokaryotes or using chemical synthesis. Such non-glycosylated products are among the peptides and proteins that are suitable bioactive components of the present invention. Finally, although some antibodies function as receptor-binding agonists or antagonists (*see, e.g.*, Morris, J.C., *et al.*, (2000) *Ann Rheum Dis 59 (Suppl I)*:i109-i114), such immunoglobulins are not suitable candidates for N-terminal polymer coupling within the scope of this invention, *i.e.*, they are not RN receptor-binding proteins, since the amino-terminal regions of both the light and heavy chains participate in antigen recognition.

[0090] Of particular use as bioactive components for use in preparing the polymer conjugates of the present invention are interferon-*alpha*, interferon-*beta* (including IFN-*beta*-1b), IL-2, IL-4, IL-10, TNF-*alpha*, hGH, prolactin, insulin, IGF-1, EGF, bFGF and erythropoietin (Epo). Also of particular use are muteins and fragments of such bioactive components, particularly those capable of binding to the receptors for the corresponding wild-type or intact polypeptide, whether or not this binding induces a biological or physiological effect. In certain such embodiments, muteins and fragments of the bioactive

components can act as antagonists for the corresponding ligands, which reduce, substantially reduce or completely inhibit the binding of ligands to their receptors and/or the activity of the ligands on their target cells, tissues and/or organisms. Other antagonists, which may or may not be structural analogues, muteins, variants or derivatives of the ligands of interest, are also suitable for preparation of the conjugates in accordance with the present invention. As a practical matter, whether or not a given mutein, fragment, variant, derivative or antagonist antagonizes the biological and/or physiological effects of a given ligand can be determined, without undue experimentation, using assays for the biological/physiological effects of the ligand itself, a variety of which are well-known in the art and/or described herein.

[0091] The structures (primary, secondary, tertiary and, where applicable, quaternary) for these and other polypeptides of interest that are advantageously used in accordance with the present invention are well-known in the art and will be familiar to one of ordinary skill, particularly in view of the structures provided herein and in the references cited herein, which are incorporated herein by reference in their entireties.

Conjugates

[0092] The present invention provides stable conjugates of bioactive components, particularly of cytokines, chemokines, growth factors, and polypeptide hormones, for use in a variety of applications. Such conjugates of the invention have a number of advantages over those previously known in the art, as shown by the following non-limiting and exemplary comparisons of art-known conjugates:

[0093] H. Hiratani (European Patent No. EP 0 098 110 and U.S. Patent No. 4,609,546) discloses conjugates of copolymers of ethylene oxide and propylene oxide ("PEG-PPG," a member of the general class of PAGs) with proteins, including interferons and interleukins, wherein no preference for avoiding regions of the proteins involved in receptor binding is disclosed. In

these references, interferons *alpha*, *beta* and *gamma* were considered to be equivalent targets for coupling of PAG, unlike in the present invention wherein interferon-*gamma* is not considered to be a suitable target for N-terminal coupling because the amino terminus is within the receptor-binding region of this cytokine. In addition, Hiratani discloses conjugates synthesized only with PAGs of 1 kDa to 10 kDa, whereas the methods of the present invention prefer the coupling of water-soluble, synthetic polymers with molecular weights exceeding 10 kDa for therapeutic applications. Analogously, N.V. Katre ((1990) *J Immunol 144*:209-213) discloses that coupling larger numbers of strands of 5-kDa mPEG to human recombinant interleukin-2 increases the life-times of the resultant conjugates in the bloodstreams of mice and rabbits. However, this reference did not disclose or recognize the advantage of coupling a smaller number of longer strands of PEG or of coupling a single strand of high molecular weight PEG to the amino terminus of IL-2, as provided by the present invention.

[0094] G. Shaw (U.S. Patent No. 4,904,584 and PCT Publication No. WO 89/05824 A2) discloses methods for inducing site-selective attachment of amine-reactive polymers by introducing, replacing or deleting lysine residues in the target protein, especially Epo, G-CSF and IL-2. However, unlike the disclosure of the present invention, these references do not disclose that amine-reactive polymers can react with any amine in the target protein other than the *epsilon* amino groups of lysine residues, clearly distinguishing these disclosures from the present invention.

[0095] D.E. Nitecki *et al.*, (U.S. Patent No. 4,902,502) disclose multiply PEGylated IL-2 conjugates that were prepared from various chloroformate derivatives of PEG that were intended to react with the *epsilon* amino groups of lysine residues. In contrast to the present methods, however, this reference discloses no method to avoid PEGylation of lysine residues in regions of the IL-2 protein that are involved in receptor binding, nor any awareness that avoidance of such sites is advantageous.

[0096] N. Katre *et al.*, (U.S. Patent No. 5,206,344) disclose PEG-IL-2 conjugates in which PEG is coupled to the *epsilon* amino groups of lysine residues, to the unpaired sulfhydryl group of the naturally occurring cysteine residue at position 125 (counting from the amino terminus) or to the sulfhydryl group of a cysteine residue that has been mutationally introduced between the first and twentieth residues from the amino terminus of IL-2. Included among the muteins that are disclosed in the '344 patent is "*des*-ala-1" IL-2, *i.e.*, a mutein in which the amino-terminal alanine is deleted and not PEGylated. In contrast to the present disclosure, however, the '344 patent does not disclose any method for avoiding coupling PEG to amino acid residues that are involved in binding to receptors, nor any recognition that such an approach would be advantageous. Consistent with this notion, and in contrast to the present invention, the broad range of points of attachment proposed in the '344 patent does not suggest that coupling PEG to the amino terminus of IL-2 would be especially advantageous.

[0097] S.P. Monkarsh *et al.*, (1997) *Anal Biochem 247*:434-440 and S.P. Monkarsh *et al.*, (1997) *in* Harris, J.M., *et al.*, eds., *Poly(ethylene glycol): Chemistry and Biological Applications*, pp. 207-216, American Chemical Society, Washington, D.C., disclose that reacting interferon-*alpha*-2a with a three-fold molar excess of an activated PEG with a molecular weight of 5,300 Daltons produces eleven positional isomers of monoPEG-interferon, corresponding to the eleven lysine residues in interferon-*alpha*-2a. No PEG-interferon in which the PEG is coupled to the *alpha* amino group at the amino terminus of the interferon was reported. The eleven positional isomers reported in these references displayed antiviral activities in cell cultures that ranged from 6% to 40% of that of the unmodified interferon and antiproliferative activities in cell cultures that ranged from 9% to 29% of that of the unmodified interferon. Such results clearly demonstrate that the random PEGylation of lysine residues practiced by these investigators interfered with the functions of interferon-*alpha*-2a mediated by its receptors, in contrast to conjugates prepared by the methods of the present invention. In addition,

unlike the conjugates of the present invention, there was no N-terminally PEGylated interferon in the conjugates reported in these references.

[0098] O. Nishimura *et al.*, (U.S. Patent Statutory Invention Registration No. H1662) disclose conjugates of interferon-*alpha*, interferon-*gamma* and IL-2 that are prepared by reductive alkylation of activated "polyethylene glycol methyl ether aldehydes" with sodium cyanoborohydride at pH 7.0 (for the interferon conjugates) or pH 7.15 (for the IL-2 conjugates). The conjugates prepared by such methods, however, were reported to have lost up to 95% of the bioactivity of the unmodified proteins, apparently due to the presence of multiple sites of polymer attachment, all of which were reported to be on the *epsilon* amino groups of lysine residues (*cf.*, Figures 1 and 4 of the present invention).

[0099] D.K. Pettit *et al.*, *supra*, disclose polymer conjugates of interleukin-15 ("IL-15"). The conjugated IL-15 reported in this reference, however, not only lost its IL-2-like growth-promoting capacity as a result of coupling polymers to lysine residues in regions of the protein that are involved in receptor binding, but it also showed antagonism rather than agonism. These authors conclude that selective inhibition of binding of IL-15 to one of several cell surface receptors can be a consequence of polymer conjugation and that such inhibition can not only decrease receptor binding, but can reverse the biological effect of the protein. By avoiding the coupling of polymers to portions of the receptor-binding protein that are involved in interactions with their receptors, the present invention avoids this undesirable consequence of polymer coupling.

[00100] J. Hakimi *et al.*, (U.S. Patent Nos. 5,792,834 and 5,834,594) disclose urethane-linked PEG conjugates of proteins, including interferon-*alpha*, IL-2, interleukin-1 ("IL-1") and an antagonist of the IL-1-receptor, which were reportedly prepared in order to decrease the immunogenicity, increase the solubility and increase the biological half-life of the respective proteins. In these references, PEG was coupled to "various free amino groups," with no reference to N-terminal PEGylation and no disclosure that the N-terminal

alpha amino groups could or should be PEGylated. These patents also state that the conjugate disclosed therein "has at least a portion" of the original biological activity of the starting protein, thus indicating possible loss of substantial bioactivity. This result would be consistent with the use of the untargeted PEGylation methods disclosed therein. In contrast to the present invention, these patents do not disclose any attempt to improve the retention of bioactivity of their conjugates by altering the selectivity of the PEGylation processes disclosed therein.

[00101] O.B. Kinstler *et al.*, (European Patent Publication No. EP 0 822 199 A2) disclose a process for reacting poly(ethylene glycol) with the *alpha* amino group of the amino acid at the amino terminus of a polypeptide, especially consensus interferon and G-CSF, which are two of the proteins manufactured by Amgen, Inc., the assignee of this patent application. This publication indicates that "a pH sufficiently acidic to selectively activate the *alpha* amino group" is a necessary feature of the disclosed process. In contrast, by the present invention it has been discovered that lowering the pH *decreases* the reactivity of amino groups with PEG aldehydes and that the *alpha* amino group is more reactive when it is *not* protonated, *i.e.,* at a pH above its pK_a. Thus, the present inventors find that no pH is "sufficiently acidic to selectively activate the *alpha* amino group" of any of the RN cytokines of the present invention. The explanations of the pH dependence of the reactivity of N-terminal *alpha* amino groups with aldehydes given by J.T. Edsall (*supra*) and by R.S. Larsen *et al.*, ((2001) *Bioconjug Chem 12*:861-869) are more compatible with the experience of the present inventors. Furthermore, Kinstler *et al.* report the use of N-terminal PEGylation of polypeptides for increased homogeneity of the resulting conjugates and protection of the amino terminus from degradation by proteinases, but do not disclose that N-terminal PEGylation can preserve a greater fraction of the receptor-binding activity of certain receptor-binding proteins (*see, e.g.*, PCT Publication No. WO 96/11953; European Patent No. EP 0 733 067, and U.S. Patent Nos. 5,770,577, 5,824,784 and 5,985,265, all of Kinstler, O.B., *et al.*).

[00102] The European application of Kinstler *et al.,* (EP 0 822 199 A2) also generalizes the benefits of N-terminal PEGylation to all polypeptides, which has not been the experience of the present inventors. Specifically, since the amino termini of antibody molecules occur proximal to the antigen-combining region of the antibody proteins (Chapman, A.P. (2002) *Adv Drug Deliv Rev 54*:531-545), N-terminal PEGylation of antibodies is unexpectedly deleterious to bioactivity, compared to random PEGylation of lysine residues, as disclosed by Larsen, R.S., *et al.*, *supra*. Similarly, N-terminal PEGylation of receptor-binding proteins that are not "RN" receptor-binding proteins, *e.g.*, interferon-*gamma* (see Figure 8), is expected to be more inhibitory of interactions with receptors than random PEGylation of the lysine residues of such receptor-binding proteins.

[00103] Thus, as noted above, the methods of the present invention are distinguished from those disclosed by Kinstler *et al.* in the publications cited herein, in that the conjugates of the present invention are prepared by conjugating one or more cytokines, chemokines, growth factors, polypeptide hormones or antagonists thereof that are selected as RN receptor-binding proteins with one or more polymers by forming a mixture between the ligand(s) and the one or more polymers at a pH of about 5.6 to about 7.6; at a pH of about 5.6 to about 7.0; at a pH of about 6.0 to about 7.0; at a pH of about 6.5 to about 7.0; at a pH of about 6.6 to about 7.6; at a pH of about 6.6 to about 7.0; or at a pH of about 6.6. In contrast, the methods of Kinstler *et al.* rely on conjugation of ligands at a pH below 5.5, which pH range the present inventors have found to be suboptimal or inferior for preparing preparations of ligands selectively conjugated with polymers at remote N-terminal amino acids and/or at remote glycosylation sites.

[00104] R.B. Pepinsky *et al.*, (PCT Publication No. WO 00/23114 and U.S. Patent Application Publication No. 2003/0021765 A1) disclose polymer conjugates of glycosylated interferon-*beta*-1a that are more active than nonglycosylated interferon-*beta*-1b in an antiviral assay. This reference also discloses that polyalkylene glycol can be coupled to the interferon-*beta*-1a *via*

a variety of coupling groups at various sites, including the amino terminus, the carboxyl terminus and the carbohydrate moiety of the glycosylated protein. It is not disclosed in this publication, however, that the methods described can be generalized to other proteins: "[t]hese studies indicate that, despite the conservation in sequence between interferon-*beta*-1a and interferon-*beta*-1b, they are distinct biochemical entities and therefore much of what is known about interferon-*beta*-1b cannot be applied to interferon-*beta*-1a, and vice versa." In contrast, the present invention discloses the common features embodied in "RN" and "RG" receptor-binding proteins, as defined herein. According to the present invention, both interferon-*beta*-1a and interferon-*beta*-1b are "RN" receptor-binding proteins. In addition, interferon-*beta*-1b is an "RG" receptor-binding protein. Accordingly, in contrast to the methods of WO 00/23114, the methods of the present invention are useful for preparing stable, bioactive conjugates of both interferon-*beta*-1b and interferon-*beta*-1a.

[00105] Z. Wei *et al.* (U.S. Patent No. 6,077,939) disclose methods for coupling water-soluble polymers (especially PEG) to the N-terminal *alpha* carbon atom of a polypeptide (especially erythropoietin), wherein the amine at the *alpha* carbon of the N-terminal amino acid is first transaminated to an *alpha* carbonyl group that is then reacted with a PEG derivative to form an oxime or a hydrazone bond. Since the disclosed objective of this reference was to develop a method that would be applicable to proteins in general, no consideration was given to the preservation of receptor-binding activity that can result from the choice of the amino terminus as the site of PEGylation of certain receptor-binding proteins. Thus, in contrast to the disclosure of Wei *et al.*, the present invention does not require the removal of the N-terminal *alpha* amino group, but, in contrast, can preserve the charge of the N-terminal *alpha* amino group at neutral pH through the formation of a secondary amine linkage between the protein and the polymer.

[00106] C.W. Gilbert *et al.*, (U.S. Patent No. 6,042,822; European Patent No. EP 1 039 922) disclose the desirability of a mixture of positional isomers of PEG-interferon-*alpha*-2b wherein an especially desirable isomer has PEG

coupled to a histidine residue of interferon-*alpha*-2b, especially histidine-34, and demonstrate that the PEG linkage to histidine-34 is unstable. Since histidine-34 lies on the surface of interferon-*alpha*-2b in a region that must come into intimate contact with an interferon receptor in order to trigger signal transduction (see Figure 1b of the present specification), the instability of the linkage between PEG and histidine-34 disclosed in these references appears to be critical to the function of the PEG-interferon conjugate disclosed therein. Substantially pure histidine-linked protein polymer conjugates were described by S. Lee *et al.*, U.S. Patent No. 5,985,263. In contrast, the present invention demonstrates that one preferred conjugate is a PEG-interferon conjugate wherein the PEG is stably linked at a site that is remote from the receptor-binding domains of the interferon component.

[00107] P. Bailon *et al.*, ((2001) *Bioconjug Chem 12*:195-202), disclose that interferon-*alpha*-2a that is PEGylated with one molecule of 40-kDa di-mPEG-lysine per molecule of interferon is comprised of four major positional isomers. This reference discloses that nearly all of the PEG was attached by amide bonds to lysines 31, 121, 131 or 134, each of which is within or adjacent to the receptor-binding domains of interferon-*alpha*-2a (residues 29-35 and 123-140, according to Bailon *et al.*; *see* Figure 1a of the present specification). N-terminal PEGylation was not reported by Bailon *et al.* Antiviral activity of the isolated mixture of positional isomers of PEG-interferon against Vesicular Stomatitis Virus infection of Madin-Darby bovine kidney cells *in vitro* was reported to be 7% of that of the unconjugated interferon-*alpha*-2a that was tested. The substantial loss of bioactivity that was observed for these PEG-interferon conjugates that do not include N-terminally PEGylated interferon thus clearly distinguishes the conjugates of Bailon *et al.* from those of the present invention.

[00108] R.B. Pepinsky *et al.*, ((2001) *J Pharmacol Exp Ther 297*:1059-1066), disclose synthesis of a conjugate from (1) glycosylated interferon-*beta*-1a having an N-terminal methionine residue and (2) a 20-kDa PEG-aldehyde. The conjugate, which is referred to in the reference as being monoPEGylated

at the N-terminal methionine, is said to retain full bioactivity in an antiviral assay, whereas the coupling of PEG of higher molecular weight decreased or eliminated the antiviral activity. While these authors disclose that their choice of the N-terminal site for PEGylation of glycosylated interferon-*beta*-1a was dictated by the availability of site-selective PEGylation reagents and molecular modeling, they acknowledge that "some effects are product specific." Moreover, and in contrast to the present invention, the observations reported therein were not generalized to include the class of receptor-binding proteins that are defined herein as "RN" receptor-binding proteins.

[00109] J. Burg *et al.,* (PCT Publication No. WO 01/02017 A2) disclose the production of alkoxyPEG conjugates of erythropoietin glycoproteins, wherein one to three strands of a methoxyPEG was/were reacted with sulfhydryl groups that were introduced chemically by modification of *epsilon* amino groups of lysine residues on the surface of the glycoprotein. In contrast to the present invention, however, this reference does not disclose any attempt to couple PEG to the free *alpha* amino group of the N-terminal amino acid of erythropoietin or to avoid modifying lysine residues in regions of the erythropoietin glycoprotein that are essential for interactions with erythropoietin receptors.

[00110] J. Burg *et al.,* (PCT Publication No. WO 02/49673 A2) disclose the synthesis of N-terminally amide-linked PEG conjugates of natural and mutein erythropoietin glycoproteins by a process that employs selectively cleavable N-terminal peptide extensions that are cleaved before PEGylation and after reversible citraconylation of all *epsilon* amino groups of the lysine residues of the glycoprotein. The disclosed rationale for the multi-step process in this reference was to make the PEGylation process selective for the free *alpha* amino group of the N-terminal amino acid in order to produce homogeneous monoPEGylated conjugates, thereby avoiding the need to separate monoPEGylated conjugates from multiply PEGylated derivatives. This method differs from that of the present invention in a number of important respects, including but not limited to: (1) the approach of Burg *et al.* is limited

to erythropoietin glycoproteins to which alkoxyPEG is linked *via* amide bonds, while the present invention is applicable to a variety of bioactive components conjugated using a variety of synthetic polymers; (2) the present invention applies to both glycosylated and nonglycosylated "RN" and "RG" receptor-binding proteins, whereas Burg *et al.* disclose only the conjugation of glycoproteins; (3) the present invention encompasses both alkoxyPEGs, such as mPEG, and monofunctionally-activated hydroxyPEGs, whereas Burg *et al.* disclose only the use of alkoxyPEGs; and (4) in the present invention, secondary amine linkages between the polymer and the protein are preferred over the amide linkages used by Burg *et al.*, since the former are more stable and conserve the positive charge on the amino group. In analogous work from the same group, J. Burg *et al.*, (U.S. Patent No. 6,340,742) disclose the production of amide-linked conjugates of erythropoietin glycoproteins, wherein one to three strands of alkoxyPEG is/are linked to one to three amino groups of the protein. In contrast to the present invention, however, this reference reports no preference for the *alpha* amino group of the N-terminal amino acid or for amino groups that are not in regions that are involved in interactions with receptors.

[00111] C. Delgado *et al.*, (U.S. Patent No. 6,384,195) disclose conjugates of granulocyte-macrophage colony-stimulating factor that are prepared using a reactive polymer that is represented as tresyl monomethoxyPEG and is referred to therein as "TMPEG." This reference indicates that when TMPEG is contacted with recombinant human GM-CSF, "[t]he modified material contains species with no activity and with higher activity than unmodified material." As one of ordinary skill will readily recognize, species with no activity are undesirable in a mixture of polymer-bioactive component conjugates, particularly in compositions for therapeutic use that comprise such conjugates, since they can contribute to the risks of administering the conjugate to a patient in need of such administration without contributing to the beneficial effects. As noted herein, the present invention overcomes this limitation in the art at least in part by avoiding modification of GM-CSF and

other receptor-binding proteins at sites on the proteins that are involved in its receptor-binding activity, thereby reducing or eliminating the synthesis of species with no activity. The present invention also provides methods for the fractionation and purification of conjugates that have different sizes, different charges and/or different extents of shielding of charges on the protein by the polymer (see Figures 9-12).

[00112] It is noteworthy that U.S. Patent No. 6,384,195 does not mention the N-terminal PEGylation of GM-CSF and therefore does not recognize the advantages of the methods of the present invention. Finally, U.S. Patent No. 6,384,195 indicates a preference for conjugates in which more than one PEG is coupled to each molecule of GM-CSF, without any consideration of where on the GM-CSF molecule those PEG molecules are attached (other than being coupled to lysine residues). By stating a preference for conjugates with up to six PEG molecules per GM-CSF, the reference thus states a preference for conjugates in which PEG might be attached to all possible lysine residues, thereby ensuring that PEG will be attached in positions that sterically hinder close approach of the protein to its cell-surface receptors (see Figure 3 of the present specification). By contrast, the present invention indicates the undesirability of coupling PEG to lysine residues, except when those lysine residues are remote from the domains of the receptor-binding protein that are essential for interactions with the receptors and hence for signal transduction (in the case of agonists) or in order to competitively inhibit signal transduction (in the case of antagonists).

[00113] T. Nakamura *et al.*, (PCT Publication No. WO 02/32957 A1) discloses that increasing the molecular weight of PEG that is coupled to the *epsilon* amino group of the lysine residue at position 52 of erythropoietin glycoprotein increases the erythropoietic effect of the conjugate *in vivo* while decreasing the affinity of the conjugate for erythropoietin receptors. In contrast to the present invention, however, this reference does not disclose the coupling of PEG at the amino terminus or near a glycosylation site, nor does it recognize any advantage to doing so.

[00114] Hence, the present invention provides conjugates and methods for the synthesis of conjugates of bioactive components coupled to synthetic polymers that have distinct structural and functional advantages over those that have been previously disclosed.

Compositions

[00115] The invention provides conjugates or complexes comprising one or more bioactive components, suitably one or more cytokines, chemokines, growth factors or polypeptide hormones, coupled to one or more stabilizing polymers such as one or more PEGs. Typically, such conjugates are produced by the methods of the present invention described herein; however, conjugates having structures and activities other than those described herein are considered equivalent if they are produced by the present methods, and are therefore encompassed by the present invention. In related aspects, the invention also provides compositions comprising one or more such conjugates or complexes. Compositions according to this aspect of the invention will comprise one or more (*e.g.*, one, two, three, four, five, ten, *etc.*) of the above-described conjugates or complexes of the invention. In certain such aspects, the compositions may comprise one or more additional components, such as one or more buffer salts, one or more chaotropic agents, one or more detergents, one or more proteins (*e.g.*, albumin or one or more enzymes), one or more unbound polymers, one or more osmotically active agents and the like. The compositions of this aspect of the invention may be in any form, including solid (*e.g.*, dry powder) or solution (particularly in the form of a physiologically compatible buffered salt solution comprising one or more of the conjugates of the invention).

A. Pharmaceutical Compositions

[00116] Certain compositions of the invention are particularly formulated for use as pharmaceutical compositions for use in prophylactic, diagnostic or therapeutic applications. Such compositions will typically comprise one or

more of the conjugates, complexes or compositions of the invention and one or more pharmaceutically acceptable carriers or excipients. The term "pharmaceutically acceptable carrier or excipient," as used herein, refers to a non-toxic solid, semisolid or liquid filler, diluent, encapsulating material or formulation auxiliary of any type that is capable of being tolerated by a recipient animal, including a human or other mammal, into which the pharmaceutical composition is introduced, without adverse effects resulting from its addition.

[00117] The pharmaceutical compositions of the invention may be administered to a recipient *via* any suitable mode of administration, such as orally, rectally, parenterally, intrasystemically, vaginally, intraperitoneally, topically (as by powders, ointments, drops or transdermal patch), buccally, as an oral or nasal spray or by inhalation. The term "parenteral" as used herein refers to modes of administration that include intravenous, intra-arterial, intramuscular, intraperitoneal, intracisternal, subcutaneous and intra-articular injection and infusion.

[00118] Pharmaceutical compositions provided by the present invention for parenteral injection can comprise pharmaceutically acceptable sterile aqueous or nonaqueous solutions, dispersions, suspensions or emulsions, as well as sterile powders for reconstitution into sterile injectable solutions or dispersions prior to use. Examples of suitable aqueous and nonaqueous carriers, diluents, solvents or vehicles include water, ethanol, polyols (such as glycerol and the like, propylene glycol, poly(ethylene glycol)), carboxymethylcellulose and suitable mixtures thereof, vegetable oils (such as olive oil), and injectable organic esters such as ethyl oleate. Proper fluidity can be maintained, for example, by the use of coating materials such as lecithin, by the maintenance of the required particle size in the case of dispersions, and by the use of surfactants.

[00119] Such pharmaceutical compositions of the present invention may also contain adjuvants such as preservatives, wetting agents, emulsifying agents and dispersing agents. Prevention of the action of microorganisms may be

ensured by the inclusion of various antibacterial and antifungal agents, for example, paraben, benzyl alcohol, chlorobutanol, phenol, sorbic acid, and the like. It may also be desirable to include osmotic agents such as sugars, sodium chloride and the like. Prolonged absorption of the injectable pharmaceutical form may be brought about by the inclusion of agents that delay absorption, such as aluminum monostearate, hydrogels and gelatin.

[00120] In some cases, in order to prolong the effect of the drugs, it is desirable to slow the absorption from subcutaneous or intramuscular injection. This may be accomplished by the use of a liquid suspension of crystalline or amorphous material with poor solubility in aqueous body fluids. The rate of absorption of the drug then depends upon its rate of dissolution, which, in turn, may depend upon its physical form. Alternatively, delayed absorption of a parenterally administered drug form can be accomplished by dissolving or suspending the drug in an oil vehicle.

[00121] Injectable depot forms are made by forming microencapsulated matrices of the drug in biodegradable polymers such as polylactide-polyglycolide. Depending upon the ratio of drug to carrier polymer and the nature of the particular carrier polymer employed, the rate of drug release can be controlled. Examples of other biodegradable polymers include biocompatible poly(orthoesters) and poly(anhydrides). Depot injectable formulations are also prepared by entrapping the drug in liposomes or microemulsions that are compatible with body tissues.

[00122] The injectable formulations can be sterilized, for example, by filtration through a bacteria-retaining filter, or by incorporating sterilizing agents in the form of sterile solid compositions that can be dissolved or dispersed in sterile water or other sterile injectable medium prior to use.

[00123] Solid dosage forms for oral administration include capsules, tablets, pills, powders and granules. In such solid dosage forms, the active compounds are mixed with at least one pharmaceutically acceptable excipient or carrier such as sodium citrate or dicalcium phosphate and/or a) fillers or extenders such as starches, lactose, sucrose, glucose, mannitol, and silicic acid,

b) binders such as, for example, carboxymethylcellulose, alginates, gelatin, polyvinylpyrrolidone, sucrose, and gum acacia, c) humectants such as glycerol, d) disintegrating agents such as agar-agar, calcium carbonate, potato or tapioca starch, alginic acid, certain silicates, and sodium carbonate, e) solution retarding agents such as paraffin, f) accelerators of absorption, such as quaternary ammonium compounds, g) wetting agents such as, for example, cetyl alcohol and glycerol monostearate, h) adsorbents such as kaolin and bentonite clay, and i) lubricants such as talc, calcium stearate, magnesium stearate, solid PEGs, sodium lauryl sulfate, and mixtures thereof. In the case of capsules, tablets and pills, the dosage form may also comprise buffering agents.

[00124] Solid compositions of a similar type may also be employed as fillers in soft- and hard-filled gelatin capsules using such excipients as lactose (milk sugar) as well as high molecular weight PEGs and the like.

[00125] The solid dosage forms of tablets, dragees, capsules, pills and granules can be prepared with coatings and shells such as enteric or chronomodulating coatings and other coatings well known in the pharmaceutical formulating art. They may optionally contain opacifying agents and can also be of such a composition that they release the active ingredient(s) only, or preferentially, in a certain part of the gastrointestinal tract, optionally, in a delayed manner. Examples of embedding compositions that can be used include polymeric substances and waxes. The active compounds can also be in microencapsulated form, if appropriate, with one or more of the above-mentioned excipients.

[00126] Liquid dosage forms for oral administration can include pharmaceutically acceptable emulsions, solutions, suspensions, syrups and elixirs. In addition to the active compounds, the liquid dosage forms may contain inert diluents commonly used in the art such as, for example, water or other solvents, solubilizing agents and emulsifiers such as ethyl alcohol, isopropyl alcohol, ethyl carbonate, ethyl acetate, benzyl alcohol, benzyl benzoate, propylene glycol, 1,3-butylene glycol, dimethyl formamide, oils (in

particular, cottonseed, groundnut, corn, germ, olive, castor, and sesame oils), glycerol, tetrahydrofurfuryl alcohol, poly(ethylene glycols) and fatty acid esters of sorbitan, and mixtures thereof.

[00127] In addition to inert diluents, the oral compositions can also include adjuvants such as wetting agents, emulsifying and suspending agents, sweetening, flavoring and perfuming agents.

[00128] Suspensions, in addition to the active compounds, may contain suspending agents as, for example, ethoxylated isostearyl alcohols, polyoxyethylene sorbitol and sorbitan esters, microcrystalline cellulose, aluminum metahydroxide, bentonite, agar-agar, tragacanth, and mixtures thereof.

[00129] Topical administration includes administration to the skin or mucosa, including surfaces of the lung and eye. Compositions for topical administration, including those for inhalation, may be prepared as a dry powder which may be pressurized or non-pressurized. In non-pressurized powder compositions, the active ingredients in finely divided form may be used in admixture with a larger-sized pharmaceutically acceptable inert carrier comprising particles having a size, for example, of up to 100 micrometers in diameter. Suitable inert carriers include sugars such as lactose and sucrose. Desirably, at least 95% by weight of the particles of the active ingredient have an effective particle size in the range of 0.01 to 10 micrometer.

[00130] Alternatively, the pharmaceutical composition may be pressurized and contain a compressed gas, such as nitrogen or a liquefied gas propellant. The liquefied propellant medium and indeed the total composition may be preferably such that the active ingredients do not dissolve therein to any substantial extent. The pressurized composition may also contain a surface-active agent. The surface-active agent may be a liquid or solid non-ionic surface-active agent or may be a solid anionic surface-active agent. It is preferable to use the solid anionic surface-active agent in the form of a sodium salt.

[00131] A further form of topical administration is to the eye. In this mode of administration, the conjugates or compositions of the invention are delivered in a pharmaceutically acceptable ophthalmic vehicle, such that the active compounds are maintained in contact with the ocular surface for a sufficient time period to allow the compounds to penetrate the conjunctiva or the corneal and internal regions of the eye, as for example the anterior chamber, posterior chamber, vitreous body, aqueous humor, vitreous humor, cornea, iris/ciliary, lens, choroid/retina and sclera. The pharmaceutically acceptable ophthalmic vehicle may, for example, be an ointment, vegetable oil or an encapsulating material.

[00132] Compositions for rectal or vaginal administration are preferably suppositories that can be prepared by mixing the conjugates or compositions of the invention with suitable non-irritating excipients or carriers such as cocoa butter, PEG or a suppository wax, which are solid at room temperature but liquid at body temperature and therefore melt in the rectum or vaginal cavity and release the drugs.

[00133] The pharmaceutical compositions used in the present therapeutic methods may also be administered in the form of liposomes. As is known in the art, liposomes are generally derived from phospholipids or other lipid substances. Liposomes are formed by mono- or multi-lamellar hydrated liquid crystals that are dispersed in an aqueous medium. Any non-toxic, physiologically acceptable and metabolizable lipid capable of forming liposomes can be used. In addition to one or more of the conjugates or compositions of the invention, the present pharmaceutical compositions in liposome form can also contain one or more stabilizers, preservatives, excipients, and the like. The preferred lipids are the phospholipids and the phosphatidyl cholines (lecithins), both natural and synthetic. Methods to form liposomes are known in the art (*see, e.g.*, Zalipsky, S., *et al.*, U.S. Patent No. 5,395,619). Liposomes that comprise phospholipids that are conjugated to PEG, most commonly phosphatidyl ethanolamine coupled to monomethoxy-

PEG, have advantageous properties, including prolonged lifetimes in the blood circulation of mammals (Fisher, D., U.S. Patent No. 6,132,763).

B. Uses

[00134] As noted elsewhere herein, the methods, conjugates and compositions of the present invention are advantageously used in methods for maintaining the bioactivity of the biological components without interfering with the ability of the biological components to bind to their receptors. Certain such methods of the invention may entail delivering one or more of the conjugates and compositions to cells, tissues, organs or organisms. In particular, the invention provides controlled delivery of the one or more components of the complexes or compositions to cells, tissues, organs or organisms, thereby providing the user with the ability to regulate, temporally and spacially, the amount of a particular component that is released for activity on the cells, tissues, organs or organisms.

[00135] In general, such methods of the invention involve one or more activities. For example, one such method of the invention comprises: (a) preparing one or more conjugates or compositions of the invention as detailed herein; and (b) contacting one or more cells, tissues, organs or organisms with the one or more conjugates or compositions, under conditions favoring the binding of the one or more conjugates or compositions of the invention to the cells, tissues, organs or organisms. Once the bioactive components of the conjugates and/or compositions of the invention have been bound by (or, in some cases, internalized by) the cells, tissues, organs or organisms, the components proceed to carry out their intended biological functions. For example, peptide components may bind to receptors or other components on or within the cells, tissues, organs or organisms; to participate in metabolic reactions within the cells, tissues, organs or organisms; to carry out, upregulate or activate, or downregulate or inhibit, one or more enzymatic activities within the cells, tissues, organs or organisms; to provide a missing structural component to the cells, tissues, organs or organisms; to provide one

or more nutritional needs to the cells, tissues, organs or organisms; to inhibit, treat, reverse or otherwise ameliorate one or more processes or symptoms of a disease or physical disorder; and the like.

[00136] In additional embodiments, the conjugates and compositions of the invention can be used in industrial cell culture, due to the unexpectedly high potencies of the bioactive components of the conjugates that are obtained as a result of the combined effects of substantial retention of their bioactivity and increased duration of action even under the harsh conditions of industrial use. These unexpectedly high potencies of the present conjugates can lead to unusually high biomass production, unusually high levels of expression of recombinant proteins, and other improvements in efficiencies of bioprocessing.

C. Dose Regimens

[00137] The conjugates, complexes or compositions of the invention can be administered *in vitro*, *ex vivo* or *in vivo* to cells, tissues, organs or organisms to deliver thereto one or more bioactive components (*i.e.*, one or more cytokines, chemokines, growth factors or polypeptide hormones or antagonists thereof). One of ordinary skill will appreciate that effective amounts of a given active compound, conjugate, complex or composition can be determined empirically and may be employed in pure form or, where such forms exist, in pharmaceutically acceptable formulation or prodrug form. The compounds, conjugates, complexes or compositions of the invention may be administered to an animal (including a mammal, such as a human) patient in need thereof as veterinary or pharmaceutical compositions in combination with one or more pharmaceutically acceptable excipients. The therapeutically effective dose level for any particular patient will depend upon a variety of factors including the type and degree of the cellular response to be achieved; the identity and/or activity of the specific compound(s), conjugate(s), complex(es) or composition(s) employed; the age, body weight or surface area, general health, gender and diet of the patient; the time of administration, route of

administration, and rate of excretion of the active compound(s); the duration of the treatment; other drugs used in combination or coincidental with the specific compound(s), conjugate(s), complex(es) or composition(s); and like factors that are well known to those of ordinary skill in the pharmaceutical and medical arts. For example, it is well within the skill of the art to start doses of a given compound, conjugate, complex or composition of the invention at levels lower than those required to achieve the desired therapeutic effect and to gradually increase the dosages until the desired effect is achieved.

[00138] Dose regimens may also be arranged in a patient-specific manner to provide a predetermined concentration of a given active compound in the blood, as determined by techniques accepted and routine in the art, *e.g.* size-exclusion, ion-exchange or reversed-phase high performance liquid chromatography ("HPLC"), bioassays or immunoassays. Thus, patient dose regimens may be adjusted to achieve relatively constant blood levels, as measured by HPLC or immunoassays, according to methods that are routine and familiar to those of ordinary skill in the medical, pharmaceutical and/or pharmacological arts.

D. Diagnostic and Therapeutic Uses

[00139] A diagnostic use of a conjugate of the invention might be for locating cells or tissues having unusually high binding capacity for the cytokine, chemokine, growth factor or polypeptide hormone, *e.g.*, a cancer, within the body of an animal, especially a human, by administration of a conjugate or composition of the invention, in which the conjugate (or one or more components, *i.e.*, the bioactive component and/or the synthetic polymer) is labeled or comprises one or more detectable labels so as to enable detection, *e.g.*, by optical, radiometric, fluorescent or resonant detection according to art-known methods. For example, the majority of non-small cell lung cancers express unusually high concentration of receptors for epidermal growth factor (Bunn, P.A., *et al.*, (2002) *Semin Oncol 29 (Suppl 14)*:38-44). Hence, in another aspect of the invention, the conjugates and compositions of the

invention may be used in diagnostic or therapeutic methods, for example in diagnosing, treating or preventing a variety of physical disorders in an animal, particularly a mammal such as a human, predisposed to or suffering from such a disorder. In such approaches, the goal of the therapy is to delay or prevent the development of the disorder, and/or to cure, induce a remission or maintain a remission of the disorder, and/or to decrease or minimize the side effects of other therapeutic regimens.

[00140] Hence, the conjugates, complexes and compositions of the present invention may be used for protection, suppression or treatment of physical disorders, such as infections or diseases. The term "protection" from a physical disorder, as used herein, encompasses "prevention," "suppression" and "treatment." "Prevention" involves the administration of a complex or composition of the invention prior to the induction of the disease or physical disorder, while "suppression" involves the administration of the conjugate or composition prior to the clinical appearance of the disease; hence, "prevention" and "suppression" of a physical disorder typically are undertaken in an animal that is predisposed to or susceptible to the disorder, but that is not yet suffering therefrom. "Treatment" of a physical disorder, however, involves administration of the therapeutic conjugate or composition of the invention after the appearance of the disease. It will be understood that in human and veterinary medicine, it is not always possible to distinguish between "preventing" and "suppressing" a physical disorder. In many cases, the ultimate inductive event or events may be unknown or latent, and neither the patient nor the physician may be aware of the inductive event until well after its occurrence. Therefore, it is common to use the term "prophylaxis," as distinct from "treatment," to encompass both "preventing" and "suppressing" as defined herein. The term "protection," used in accordance with the methods of the present invention, therefore is meant to include "prophylaxis." Methods according to this aspect of the invention may comprise one or more steps that allow the clinician to achieve the above-described therapeutic goals. One such method of the invention may comprise, for example: (a) identifying

an animal (preferably a mammal, such as a human) suffering from or predisposed to a physical disorder; and (b) administering to the animal an effective amount of one or more of the conjugates, complexes or compositions of the present invention as described herein, such that the administration of the conjugate, complex or composition prevents, delays or diagnoses the development of, or cures, induces a remission or maintains a remission of, the physical disorder in the animal.

[00141] As used herein, an animal that is "predisposed to" a physical disorder is defined as an animal that does not exhibit a plurality of overt physical symptoms of the disorder but that is genetically, physiologically or otherwise at risk for developing the disorder. In the present methods, the identification of an animal (such as a mammal, including a human) that is predisposed to, at risk for, or suffering from a given physical disorder may be accomplished according to standard art-known methods that will be familiar to the ordinarily skilled clinician, including, for example, radiological assays, biochemical assays (*e.g.*, assays of the relative levels of particular peptides, proteins, electrolytes, *etc.*, in a sample obtained from an animal), surgical methods, genetic screening, family history, physical palpation, pathological or histological tests (*e.g.*, microscopic evaluation of tissue or bodily fluid samples or smears, immunological assays, *etc.*), testing of bodily fluids (*e.g.*, blood, serum, plasma, cerebrospinal fluid, urine, saliva, semen and the like), imaging, (*e.g.*, radiologic, fluorescent, optical, resonant (*e.g.*, using nuclear magnetic resonance ("NMR") or electron spin resonance ("ESR")), *etc.* Once an animal has been identified by one or more such methods, the animal may be aggressively and/or proactively treated to prevent, suppress, delay or cure the physical disorder.

[00142] Physical disorders that can be prevented, diagnosed or treated with the conjugates, complexes, compositions and methods of the present invention include any physical disorders for which the bioactive component (typically, the cytokine, growth factor, chemokine or polypeptide hormone component or antagonist thereof) of the conjugates or compositions may be used in the

prevention, diagnosis or treatment. Such disorders include, but are not limited to, a variety of cancers (*e.g.*, breast cancers, uterine cancers, ovarian cancers, prostate cancers, testicular cancers, leukemias, lymphomas, lung cancers, neurological cancers, skin cancers, head and neck cancers, bone cancers, colon and other gastrointestinal cancers, pancreatic cancers, bladder cancers, kidney cancers and other carcinomas, sarcomas, adenomas and myelomas); iatrogenic diseases; infectious diseases (*e.g.*, bacterial diseases, fungal diseases, viral diseases (including hepatitis, diseases caused by cardiotropic viruses, HIV/AIDS, and the like), parasitic diseases, and the like); genetic disorders (*e.g.*, cystic fibrosis, amyotrophic lateral sclerosis, muscular dystrophy, Gaucher's disease, Pompe's disease, severe combined immunodeficiency disorder, dwarfism and the like), anemia, neutropenia, thrombocytopenia, hemophilia and other blood disorders; neurodegenerative disorders (*e.g.*, multiple sclerosis, Creutzfeldt-Jakob Disease, Alzheimer's disease, and the like); enzymatic disorders (*e.g.*, gout, uremia, hypercholesterolemia, and the like); disorders of uncertain or multifocal etiology (*e.g.*, cardiovascular disease, hypertension, inflammatory bowel disease and the like); autoimmune disorders (*e.g.*, systemic lupus erythematosus, rheumatoid arthritis, psoriasis, and the like) and other disorders of medical importance that will be readily familiar to the ordinarily skilled artisan. The conjugates, complexes, compositions and methods of the present invention may also be used in the prevention of disease progression, such as in chemoprevention of the progression of a premalignant lesion to a malignant lesion.

[00143] The therapeutic methods of the invention thus use one or more conjugates, complexes or compositions of the invention, or one or more of the pharmaceutical compositions of the invention, that may be administered to an animal in need thereof by a variety of routes of administration, including orally, rectally, parenterally (including intravenously, intra-arterially, intramuscularly, intraperitoneally, intracisternally, subcutaneously and intra-articular injection and infusion), intrasystemically, vaginally, intraperitoneally, topically (as by powders, ointments, drops or transdermal patch), buccally, as

an oral or nasal spray or by inhalation. By the invention, an effective amount of the conjugates, complexes or compositions can be administered *in vitro*, *ex vivo* or *in vivo* to cells or to animals suffering from or predisposed to a particular disorder, thereby preventing, delaying, diagnosing or treating the disorder in the animal. As used herein, "an effective amount of a conjugate (or complex or composition)" refers to an amount such that the conjugate (or complex or composition) carries out the biological activity of the bioactive component (*i.e.*, the cytokine, chemokine, growth factor, polypeptide hormone or antagonist thereof) of the conjugate, complex or composition, thereby preventing, delaying, diagnosing, treating or curing the physical disorder in the animal to which the conjugate, complex or composition of the invention has been administered. One of ordinary skill will appreciate that effective amounts of the conjugates, complexes or compositions of the invention can be determined empirically, according to standard methods well-known to those of ordinary skill in the pharmaceutical and medical arts; *see, e.g.*, Beers, M.H., *et al.*, eds. (1999) *Merck Manual of Diagnosis & Therapy*, 17th edition, Merck and Co., Rahway, NJ; Hardman, J.G., *et al.*, eds. (2001) *Goodman and Gilman's The Pharmacological Basis of Therapeutics*, 10th edition, McGraw-Hill Medical Publishing Division, New York; Speight, T.M., *et al.*, eds. (1997) *Avery's Drug Treatment*, 4th edition, Adis International, Aukland, New Zealand; Katzung, B.G., editor (2000) *Basic and Clinical Pharmacology*, 8th edition, Lange Medical Books/McGraw-Hill, New York; which references and references cited therein are incorporated entirely herein by reference.

[00144] It will be understood that, when administered to a human patient, the total daily, weekly or monthly dosage of the conjugates, complexes and compositions of the present invention will be decided by the attending physician within the scope of sound medical judgment. For example, satisfactory results are obtained by administration of certain of the conjugates, complexes or compositions of the invention at appropriate dosages depending on the specific bioactive compound used, which dosages will be readily familiar to the ordinarily skilled artisan or which may be readily determined

empirically using only routine experimentation. According to this aspect of the invention, the conjugates, complexes or compositions can be administered once or, in divided doses, *e.g.*, once or twice per day, or once or twice per week, or once or twice per month, *etc.* Appropriate dose regimens for various modes of administration (*e.g.*, parenteral, subcutaneous, intramuscular, intra-ocular, intranasal, *etc.*) can also be readily determined empirically, using only routine experimentation, or will be readily apparent to the ordinarily skilled artisan, depending on the identity of the bioactive component (*i.e.*, the cytokine, chemokine, growth factor, polypeptide hormone or antagonist thereof) of the conjugate, complex or composition.

[00145] In additional applications, the conjugates, complexes and compositions of the invention may be used to specifically target a diagnostic or therapeutic agent to a cell, tissue, organ or organism that expresses a receptor for, binds, incorporates or otherwise can take up, the bioactive component (*i.e.*, the cytokine, chemokine, growth factor, polypeptide hormone or antagonist thereof) of the conjugate, complex or composition. Methods according to this aspect of the invention may comprise, for example, contacting the cell, tissue, organ or organism with one or more conjugates, complexes or compositions of the invention, which additionally comprise one or more diagnostic or therapeutic agents, such that the conjugate, complex or composition is bound to or taken up by the cell, tissue, organ or organism, thereby delivering the diagnostic or therapeutic agent to the cell, tissue, organ or organism. The diagnostic or therapeutic agent used in accordance with this aspect of the invention may be, but is not limited to, at least one agent selected from a nucleic acid, an organic compound, a protein or peptide, an antibody, an enzyme, a glycoprotein, a lipoprotein, an element, a lipid, a saccharide, an isotope, a carbohydrate, an imaging agent, a detectable probe, or any combination thereof, which may be detectably labeled as described herein. A therapeutic agent used in this aspect of the present invention may have a therapeutic effect on the target cell (or tissue, organ or organism), the effect being selected from, but not limited to, correcting a defective gene or protein,

a drug action, a toxic effect, a growth stimulating effect, a growth inhibiting effect, a metabolic effect, a catabolic affect, an anabolic effect, an antiviral effect, an antifungal effect, an antibacterial effect, a hormonal effect, a neurohumoral effect, a cell differentiation stimulatory effect, a cell differentiation inhibitory effect, a neuromodulatory effect, an anti-neoplastic effect, an anti-tumor effect, an insulin stimulating or inhibiting effect, a bone marrow stimulating effect, a pluripotent stem cell stimulating effect, an immune system stimulating effect, and any other known therapeutic effect that may be provided by a therapeutic agent delivered to a cell (or tissue, organ or organism) *via* a delivery system according to this aspect of the present invention.

[00146] Such additional therapeutic agents may be selected from, but are not limited to, known and new compounds and compositions including antibiotics, steroids, cytotoxic agents, vasoactive drugs, antibodies and other therapeutic agents. Non-limiting examples of such agents include antibiotics and other drugs used in the treatment of bacterial shock, such as gentamycin, tobramycin, nafcillin, parenteral cephalosporins, *etc.*; adrenal corticosteroids and analogs thereof, such as dexamethasone, mitigate the cellular injury caused by endotoxins; vasoactive drugs, such as an *alpha* adrenergic receptor blocking agent (*e.g.*, phenoxybenzamine), a *beta* adrenergic receptor agonist (*e.g.*, isoproterenol), and dopamine.

[00147] The conjugates, complexes and compositions of the invention may also be used for diagnosis of disease and to monitor therapeutic response. In certain such methods, the conjugates, complexes or compositions of the invention may comprise one or more detectable labels (such as those described elsewhere herein). In specific such methods, these detectably labeled conjugates, complexes or compositions of the invention may be used to detect cells, tissues, organs or organisms expressing receptors for, or otherwise taking up, the bioactive component (*i.e.*, cytokine, chemokine, growth factor or polypeptide hormone or antagonist thereof) of the conjugates, complexes or compositions. In one example of such a method, the cell, tissue, organ or

organism is contacted with one or more of the conjugates, complexes or compositions of the invention under conditions that favor the binding or uptake of the conjugate by the cell, tissue or organism (*e.g.*, by binding of the conjugate to a cell-surface receptor or by pinocytosis or diffusion of the conjugate into the cell), and then detecting the conjugate bound to or incorporated into the cell using detection means specific to the label used (*e.g.*, fluorescence detection for fluorescently labeled conjugates; magnetic resonance imaging for magnetically labeled conjugates; radioimaging for radiolabeled conjugates; *etc.*). Other uses of such detectably labeled conjugates may include, for example, imaging a cell, tissue, organ or organism, or the internal structure of an animal (including a human), by administering an effective amount of a labeled form of one or more of the conjugates of the invention and measuring detectable radiation associated with the cell, tissue, organ or organism (or animal). Methods of detecting various types of labels and their uses in diagnostic and therapeutic imaging are well known to the ordinarily skilled artisan, and are described elsewhere herein.

[00148] In another aspect, the conjugates and compositions of the invention may be used in methods to modulate the concentration or activity of a specific receptor for the bioactive component of the conjugate on the surface of a cell that expresses such a receptor. By "modulating" the activity of a given receptor is meant that the conjugate, upon binding to the receptor, either activates or inhibits the physiological activity (*e.g.*, the intracellular signaling cascade) mediated through that receptor. While not intending to be bound by any particular mechanistic explanation for the regulatory activity of the conjugates of the present invention, such conjugates can antagonize the physiological activity of a cellular receptor by binding to the receptor *via* the bioactive component of the conjugate, thereby blocking the binding of the natural agonist (*e.g.*, the unconjugated bioactive component) and preventing activation of the receptor by the natural agonist, while not inducing a substantial activation of the physiological activity of the receptor itself. Methods according to this aspect of the invention may comprise one or more

steps, for example contacting the cell (which may be done *in vitro, ex vivo* or *in vivo*) with one or more of the conjugates of the invention, under conditions such that the conjugate (*i.e.*, the bioactive component portion of the conjugate) binds to a receptor for the bioactive component on the cell surface but does not substantially activate the receptor. Such methods will be useful in a variety of diagnostic, and therapeutic applications, as the ordinarily skilled artisan will readily appreciate.

Kits

[00149] The invention also provides kits comprising the conjugates and/or compositions of the invention. Such kits typically comprise a carrier, such as a box, carton, tube or the like, having in close confinement therein one or more containers, such as vials, tubes, ampules, bottles, syringes and the like, wherein a first container contains one or more of the conjugates and/or compositions of the present invention. The kits encompassed by this aspect of the present invention may further comprise one or more additional components (*e.g.*, reagents and compounds) necessary for carrying out one or more particular applications of the conjugates and compositions of the present invention, such as one or more components useful for the diagnosis, treatment or prevention of a particular disease or physical disorder (*e.g.*, one or more additional therapeutic compounds or compositions, one or more diagnostic reagents, one or more carriers or excipients, and the like), one or more additional conjugates or compositions of the invention, and the like.

[00150] It will be readily apparent to one of ordinary skill in the relevant arts that other suitable modifications and adaptations to the methods and applications described herein may be made without departing from the scope of the invention or any embodiment thereof. Having now described the present invention in detail, the same will be more clearly understood by reference to the following examples, which are included herewith for purposes of illustration only and are not intended to be limiting of the invention.

EXAMPLES

Example 1: PEG-Interferon-*alpha* Conjugates

[00151] Interferon-*alpha* is a commercially important medicinal protein with a world market in the year 2001 exceeding U.S. $2 billion, primarily for the treatment of patients with hepatitis C virus ("HCV") infections. In the United States, between three and four million people are infected with chronic hepatitis C and about 10,000 HCV-related deaths occur each year (Chander, G., *et al.*, (2002) *Hepatology 36*:5135-5144). In attempting to improve the usefulness of IFN-*alpha*, both of the companies that are primarily responsible for its development and marketing (Schering-Plough Corp. and F. Hoffmann-La Roche AG) have developed and commercially launched conjugates of IFN-*alpha* with monomethoxypoly(ethylene glycol) or "mPEG." In each case, mPEG is linked to each molecule of interferon-*alpha* at only one point of attachment. In each case, the product contains a mixture of positional isomers with markedly reduced receptor-binding activity, compared to the unmodified interferon. In each case, the increased bioavailability and duration of action of the conjugate *in vivo* more than compensates for the decreased bioactivity *in vitro* that results from PEG conjugation, as measured by improved clinical effectiveness of one injection of the conjugate per week, compared to three injections of the unmodified protein per week, for the treatment of chronic infection with HCV (Manns, M.P., *et al.*, (2001) *Lancet 358*:958-965).

[00152] In the PEG-interferon-*alpha*-2a conjugate of F. Hoffmann-La Roche, PEGASYS®, two strands of 20-kDa mPEG are coupled to a single lysine linker (so-called "branched PEG") that is linked primarily to one of Lys 31, Lys 121, Lys 131 or Lys 134 (Bailon, P., *et al.*, *supra*), all of which are within or adjacent to a receptor-binding domain of interferon-*alpha*-2a (see Binding Site 1 in Figure 1a and SEQ ID NO:1).

[00153] In the PEG-interferon-*alpha*-2b conjugate of Schering-Plough Corp., a single strand of 12-kDa mPEG is coupled predominantly to a histidine residue at position 34 (His 34; Wylie, D.C., *et al.*, *supra;* Gilbert, C.W., *et al.*, U.S.

Patent No. 6,042,822; Wang, Y.-S., *et al.*, *supra*), which is in a region that is important for binding to a receptor (see Figure 1b). Other sites of PEG attachment in the PEG- INTRON product of Schering-Plough (Lys 121, Tyr 129 and Lys 131) are also seen to be in or near Binding Site 1 (Figure 1b and SEQ ID NO:2).

[00154] In contrast to these two commercial products, the conjugate of the present invention has a single strand of water soluble, synthetic polymer, preferably PEG or mPEG, linked to the N-terminal amino acid residue, which is remote from the receptor-binding regions of the protein (see the spatial relationship between Cys-1 and the Binding Sites in Figures 1c and 1d), demonstrating that interferon-*alpha* is an "RN" cytokine. Figures 9 and 10 show cation-exchange and size-exclusion chromatograms, respectively, of an exemplary PEG-interferon-*alpha* conjugate of the present invention. The reaction mixture contained interferon-*alpha*-2b in which an additional methionine residue was present at the amino terminus, preceding Cys-1, which is the first residue of the natural sequence. The reactive PEG was 20-kDa PEG-aldehyde, which was present at a concentration of 0.2 mM. The reducing agent was sodium cyanoborohydride, at a final concentration of 14 mM. Progress of the reaction was monitored periodically by size-exclusion chromatography during incubation at 4°C. Although IFN-*alpha* was sufficiently soluble to be PEGylated under the conditions described, other cytokines, *e.g.*, IFN-*beta*, are less soluble and may need to be PEGylated in the presence of a surfactant, as described for IFN-*alpha* by C.W. Gilbert *et al.*, (U.S. Patent No. 5,711,944) and for interferons *alpha* and *beta* by R.B. Greenwald *et al.*, (U.S. Patent No. 5,738,846).

[00155] The cation-exchange column used for the fractionation shown in Figure 9 was ToyoPearl MD-G SP (1 x 6.8 cm; Tosoh Biosep, Montgomery-ville, PA), developed with a linear gradient of 0-0.4 M NaCl in 20 mM sodium acetate buffer, pH 4.6, at a flow rate of 0.5 mL/minute. The size-exclusion column used to obtain the data in Figure 10 was Superdex® 200 (HR 10/30; Amersham Biosciences, Piscataway, NJ), eluted at 0.5 mL/minute in 20 mM

sodium acetate buffer containing 150 mM NaCl, pH 4.6. Other suitable ion-exchange and size-exclusion chromatographic media and fractionation conditions are known to those skilled in the art. Amino-terminal amino acid analysis by automated Edman degradation of the purified monoPEG-IFN-*alpha*-2b of this invention demonstrated that greater than 90% of the PEG was attached to the N-terminal residue. The analysis was performed by Commonwealth Biotechnologies, Inc. (Richmond, VA).

Example 2: **PEG-Interleukin-2 Conjugates**

[00156] Interleukin-2 ("IL-2") is a cytokine that displays immunomodulatory activity against certain cancers, including renal cell carcinoma and malignant melanoma. However, clinical efficacy is poor, with the result that only a small fraction of patients experience partial or complete responses (Weinreich, D.M., *et al.*, (2002) *J Immunother 25*:185-187). IL-2 has a short half-life in the bloodstream, which is implicated in its low rate of induction of remission in cancer patients. Attempts to make IL-2 more useful by random PEGylation of lysine residues have not been optimal (Chen, S.A., *et al.*, (2000) *J Pharmacol Exp Ther 293*:248-259). Attempts to selectively attach PEG to IL-2 at its glycosylation site (Goodson, R.J., *et al.*, *supra*) or at a non-essential cysteine (Cys 125) or to muteins of IL-2 containing cysteine between residues 1 and 20 (Katre, N., *et al.*, U.S. Patent No. 5,206,344) have not led to clinically useful products.

[00157] Figure 4 shows the distribution of lysine residues with respect to the receptor-binding regions of IL-2, showing that many of the surface-accessible lysine residues are in regions that are involved in receptor binding. In fact, Lys-35 and Lys-43 have been identified as required for interaction with the *alpha*-receptor for IL-2, suggesting a mechanism for the inactivation of IL-2 by PEGylation of lysine residues (see SEQ ID NO:6). Figure 4 also shows that the N-terminal region of IL-2 is remote from the receptor-binding regions of the protein, demonstrating that IL-2 has the structure of an "RN" cytokine. Our conclusion that IL-2 is an "RN" cytokine is compatible with the

observations of H. Sato, *et al.*, ((2000) *Bioconjug Chem 11*:502-509), who employed enzymatic transglutamination to couple one or two strands of 10-kDa mPEG to one or two of the glutamine residues ("Q") in the sequence AQQIVM that those authors introduced into an IL-2 mutein as an N-terminal extension. Sato *et al.* reported that their conjugate that was PEGylated near the amino terminus by transglutamination of their mutein retained more bioactivity than a conjugate prepared by random PEGylation of lysines in the IL-2 mutein. For a review of analogous approaches to PEGylation of other proteins, *see* Sato, H., (2002) *supra*. Based on the spatial separation of the amino terminus of IL-2 from the receptor-binding regions of the protein, as shown in Figure 4, one can understand that the glycosylation site at residue Thr-3 (not shown) renders IL-2 an "RG" receptor-binding protein, as defined herein. Thus, IL-2 is both an RN cytokine and an RG cytokine.

[00158] Figures 11 and 12 show cation-exchange and size-exclusion chromatograms, respectively, of an exemplary PEG-IL-2 conjugate of the present invention, which was PEGylated by N-terminally selective, reductive alkylation, as in Example 1. The conditions used for fractionation were the same as those described for Figures 9 and 10, respectively. Figure 13 shows a polyacrylamide gel electrophoretic analysis of the same conjugate in the presence of sodium dodecyl sulfate ("SDS-PAGE"), before and after its purification by ion-exchange chromatography, as shown in Figure 11. The gel contained a gradient of 4-12% total acrylamide in Bis-Tris buffer (Catalog # NP0335, Invitrogen, Carlsbad, CA). The samples, each containing about 1-2 mcg protein, were heated at 90°C for 10 minutes prior to analysis. The gel was run at a constant voltage of 117-120 for about 135 minutes, with cooling. One portion of the gel was stained with Sypro® Ruby protein gel stain (Molecular Probes, Eugene, OR) and the other portion was stained for PEG by an adaptation of the methods of C.E. Childs ((1975) *Microchem J 20*:190-192) and B. Skoog ((1979) *Vox Sang 37*:345-349). Amino-terminal amino acid analysis by automated Edman degradation of the purified monoPEG-IL-2 in each of the two peaks in Figure 11 demonstrated that greater than 90% of the

PEG was attached to the N-terminal residue. The analysis was performed by Commonwealth Biotechnologies, Inc. (Richmond, VA).

Example 3: Synthesis and Analysis of N-terminally PEGylated EGF and IGF-1

[00158] Epidermal growth factor ("EGF;" SEQ ID NO:7) and insulin-like growth factor-1 ("IGF-1;" SEQ ID NO:9) were selected for N-terminal PEGylation on the basis of the molecular models in Figures 5 and 7, respectively, which showed that EGF and IGF-1 are RN growth factors. A 3 mM solution of 5-kDa PEG-aldehyde was prepared by dissolving 5-kDa PEG-propionaldehyde (NOF Corporation, Tokyo) in 1 mM HCl at a final concentration of 15 mg/mL. Borane-pyridine was prepared by dilution of 35 microliters (mcL) of 8 M borane-pyridine (Aldrich) in 0.3 mL acetonitrile plus 0.15 mL water, to give a final concentration of 0.58 M. A buffer containing 0.2 M each of sodium phosphate and sodium acetate, pH 6.3, was prepared and filtered through a 0.1-micron pore sterile filter. Recombinant human EGF from Invitrogen Corp. (Carlsbad, CA) was dissolved in water at a concentration of 1 mg/mL. To 0.6 mL of this solution, 70 mcL of 3 mM PEG-aldehyde solution, 35 mcL of phosphate-acetate buffer and 30 mcL of 0.58 M borane-pyridine solution were added and the mixture was refrigerated. Aliquots were analyzed by size-exclusion HPLC on a Superdex 75 HR 10/30 column in sodium carbonate buffer, pH 10.1, containing 100 mM NaCl after four days of incubation at 4-8°C and the eluate was monitored by absorbance at 280 nm and by refractive index. After injection of 0.65 mL of reaction mixture that had been incubated for 5 days, fractions were collected from the center of the major peak of absorbance at 280 nm. The pH of this pool was lowered to approximately 5.5 by the addition of acetic acid. Reanalysis of this pool of product by size-exclusion HPLC indicated that 100% of the protein was in the position corresponding to PEG_1-EGF ("mono-PEG-EGF") and that the protein concentration of this pool was about 0.32 mg/mL. Analysis by SDS-PAGE confirmed that all of the protein consisted of a mono-PEG

conjugate of EGF. The product pool was sterile-filtered through a 0.2-micron pore Corning syringe filter before being tested in a cell-based bioassay, as described in Example 4. The 10-kDa PEG conjugate of EGF was synthesized, purified and analyzed by a similar method, except that 10-kDa PEG-propionaldehyde from NOF Corporation was used instead of 5-kDa PEG-aldehyde. The final protein concentration of the 10-kDa PEG conjugate was about 0.36 mg/mL.

[00159] Samples of recombinant human insulin-like growth factor-1 ("IGF-1") from Invitrogen Corp. were coupled to 5-kDa or to 10-kDa PEG-aldehyde by the methods described for the corresponding EGF conjugates. The product of coupling 5-kDa PEG-aldehyde to IGF-1 and purification of the conjugate as described for PEG-EGF was about 99% pure mono-PEG-IGF-1 conjugate and the final protein concentration was about 0.20 mg/mL. SDS-PAGE analyses confirmed that the protein was predominantly in a mono-PEG conjugate. Electrophoretic analysis also revealed the presence of traces of di-PEG conjugate when the load on the gel was high. Size-exclusion HPLC analysis of the product of coupling 10-kDa PEG-aldehyde to IGF-1 indicated that the product consisted of 95% mono-PEG conjugate and about 5% di-PEG conjugate and had a total protein concentration of about 0.23 mg/mL.

Example 4: **Bioassays of N-terminally PEGylated EGF and IGF-1**

[00160] Evaluation of whether N-terminal PEGylation of EGF and IGF-1 decreases the receptor-binding capacity of the respective growth factors is performed by cell culture assays. For assays of PEG-EGF, 3T3 fibroblasts are used, as described previously for EGF (Crouch, M.F., *et al.*, (2001) *J Cell Biol 152*:263-273). For assays of PEG-IGF-1, Chinese hamster ovary ("CHO") cells are used, as described previously for IGF-1 (Amoui, M., *et al.*, (2001) *J Endocrinol 171*:153-162; Morris, A.E., *et al.*, (2000) *Biotechnol Prog 16*:693-697). Product pools of PEG-EGF and PEG-IGF-1, prepared as described in Example 3, are sterile-filtered through a 0.2-micron pore Corning syringe filter and are then tested in a cell-based bioassay. Serial dilutions of

sterile-filtered EGF and of the mono-PEG conjugates synthesized with 5-kDa and 10-kDa PEG are added to cultures of 3T3 cells in medium containing a lower percentage of serum than that required for optimal growth. The cells are cultured under standard conditions (37°C, 5% CO_2/air), and counted with a Coulter counter (Model Z1, Miami, FL) at several intervals during one week. Relative to the number of cells observed in the absence of added growth factor, the numbers of cells are increased by at least the same percentage by the mono-PEG conjugates of this invention as by unmodified EGF. Similarly, serial dilutions of the sterile-filtered mono-PEG conjugates of IGF-1 and of unmodified IGF-1 are added to cultures of CHO cells in medium containing a lower percentage of serum than that required for optimal growth, and cells are incubated and counted as described above for EGF test cultures. As observed for EGF and its N-terminal mono-PEG conjugates, the numbers of cells observed after several days are increased by at least the same percentage by the mono-PEG conjugates of IGF-1 as by the unmodified growth factor. Thus, both EGF and IGF-1 are demonstrated to be fully functional after N-terminal PEGylation, as expected for proteins that have PEG attached to the amino-terminal residue that is remote from the receptor-binding regions.

Example 5: Members and Non-members of the Class of "RN" Receptor-Binding Proteins

[00161] Figures 2, 3 and 5-8 show the surface distributions of lysine residues of the receptor-binding proteins interferon-*beta*, granulocyte-macrophage colony-stimulating factor ("GM-CSF"), epidermal growth factor ("EGF"), basic fibroblast growth factor ("bFGF," which is also known in the art as "FGF-2"), insulin-like growth factor-1 ("IGF-1") and interferon-*gamma* ("IFN-*gamma*") relative to their receptor-binding regions, as well as showing which of these proteins are "RN" cytokines and growth factors. In addition, Figure 2 shows that interferon-*beta* is an "RG" cytokine.

[00162] Figure 2 shows lysine residues distributed throughout the regions of Binding Site 1 and Binding Site 2 of interferon-*beta*, whereas the amino

terminus of the polypeptide chain is remote from the receptor-binding regions of the protein, demonstrating that IFN-*beta* is an RN cytokine (See SEQ ID NO:3).

[00163] Figure 3 shows lysine residues distributed throughout the regions of Binding Site 1, which binds the *alpha* receptor, and Binding Site 2, which binds the *beta* receptor, of GM-CSF, whereas the amino terminus of the polypeptide chain is remote from the receptor-binding regions of the protein, demonstrating that GM-CSF is an RN cytokine (See SEQ ID NO:5).

[00164] Figure 5 shows lysine residues distributed along the polypeptide chain of epidermal growth factor ("EGF"), including lysine residues that are in or near receptor-binding regions of the protein, whereas the amino terminus of the polypeptide chain is more remote from the receptor-binding regions of the protein (See SEQ ID NO:7).

[00165] Figure 6 shows that several lysine residues of basic fibroblast growth factor ("bFGF") are implicated in binding to receptors or to heparin, both of which are necessary for signal transduction by bFGF (Schlessinger, J., *et al.*, *supra*). The amino terminus of bFGF is remote from the heparin-binding region of bFGF and may be sufficiently remote from receptor binding sites to render bFGF an RN growth factor (See SEQ ID NO:8).

[00166] Figure 7 shows that several lysine residues of insulin-like growth factor-1 ("IGF-1") are within or adjacent to the receptor-binding regions of the polypeptide, whereas the amino terminus of IGF-1 is remote from the receptor-binding domains, demonstrating that IGF-1 is an RN growth factor (See SEQ ID NO:9).

[00167] Figure 8 shows that interferon-*gamma* ("IFN-*gamma*") exists as a homodimer in which the two polypeptide chains have extensive interactions. Several lysine residues of each polypeptide are adjacent to amino acid residues of IFN-*gamma* that have been implicated in binding to receptors or are in the dimerization interface. The "ball-and-stick" format of amino acid residue Gln-1 is intended to reflect the evidence for the functional importance of this N-terminal residue. The crystal structure on which this figure is based

included an additional methionine residue, labeled "Met 0," that it is not present in the natural protein (See SEQ ID NO:4). Since the N-terminal residues of IFN-*gamma* are remote from the dimerization interface, N-terminal PEGylation could avoid the inhibitory effects of lysine PEGylation on homodimerization of IFN-*gamma*. On the other hand, the interactions of the dimer with its receptors are likely to be inhibited by coupling polymers to the amino terminus, particularly when long strands of polymer are attached.

[00168] IFN-*gamma*, IL-10 and stem cell factor are examples of cytokines that function as homodimers (Walter, M.R., *et al.*, *supra*; Josephson, K., *et al.*, (2000) *J Biol Chem 275*:13552-13557; Thiel, D.J., *et al.*, *supra*; McNiece, I.K., *et al.*, *supra*). Dimerization of receptor-binding proteins presents special issues for the characterization of their N-terminally monoPEGylated conjugates, since different possible molecular structures can be present in preparations of conjugates with similar or identical size and shape. For example, a dimer that consists of one diPEGylated monomer and one unPEGylated monomer (PEG_2-$protein_1$ + $protein_1$) would be difficult or impossible to distinguish from a dimer that consist of two N-terminally PEGylated monomers (PEG_1-$protein_1)_2$ by most size-based analyses of the dimeric conjugate (*e.g.*, size-exclusion chromatography or evaluation of the sedimentation coefficient, light scattering or diffusion coefficient), yet the receptor-binding potency of these two conjugates, each containing an average of one PEG per protein monomer, might be quite different.

[00169] For the long-chain *beta*-sheet receptor-binding proteins that form homotrimers, *e.g.* tumor necrosis factor *alpha* ("TNF-*alpha*"), the number of isomers of PEG_3-$protein_3$ trimers is even larger than for the receptor-binding proteins that occur in solution as homodimers. Since chemical modification of TNF close to the amino terminus has been shown to inactivate this cytokine (Utsumi, T., *et al.*, (1992) *Mol Immunol 29*:77-81), TNF-*alpha* may not retain substantial activity when PEGylated with reagents and under certain conditions that are selective for the N-terminal residue. Nevertheless, a TNF-*alpha* antagonist such as Apo2L/TRAIL (Hymowitz, S.G. *et al.* (2000),

Biochemistry 39:633-640) is suitable for PEGylation using the present invention.

[00170] For the characterization of conjugates of cytokines that function as oligomers, a combination of analytical methods is required. Amino-terminal sequence analysis can detect the presence of monomers with free N-terminal *alpha* amino groups and electrophoretic analysis of dissociated monomers (*e.g.* SDS-PAGE or capillary electrophoresis) can reveal the presence of unPEGylated and multiply-PEGylated monomers of the receptor-binding proteins. Without such evidence, the synthesis of monoPEGylated conjugates of such homodimer- and homotrimer-forming proteins cannot be demonstrated unequivocally.

[00171] These examples, especially as graphically illustrated by Figures 1-8, provide a readily visualized basis for understanding the potential role of steric hindrance of protein-receptor interactions by PEGylation of receptor-binding proteins within or adjacent to receptor-binding domains of these bioactive components. The large volume that is occupied by the highly extended and flexible PEG strands (see Figure 1d) also would sterically hinder the association of monomers of certain receptor-binding proteins into functional homodimers or homotrimers, if the PEG were coupled in regions that are required for interactions between the monomers. Thus, the targeting of PEGylation to sites that are remote from receptor-binding regions of receptor-binding proteins decreases the likelihood that PEGylation will interfere with the intermolecular interactions that are required for their function. By proceeding in accordance with the method of this invention, more of the benefits that are expected from PEGylation of receptor-binding proteins can be realized. The resulting conjugates combine the expected benefits of improved solubility, increased bioavailability, greater stability and decreased immunogenicity with an unexpectedly high retention of bioactivity.

[00172] This invention is described with reference to certain embodiments and certain examples thereof. The methods of this invention are similarly applicable to certain receptor-binding peptides and proteins other than

cytokines, chemokines, growth factors and polypeptide hormones or their antagonists and to other conjugation reagents. Therefore, the scope of this invention is not limited to the embodiments described, but is limited only by the scope of the claims. Workers of ordinary skill in the art can readily appreciate that other embodiments can be practiced without departing from the scope of this invention. All such variations are considered to be part of this invention.

[00173] All publications, patents and patent applications mentioned in this specification are indicative of the level of skill of those skilled in the art to which this invention pertains, and are herein incorporated by reference to the same extent as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated by reference.